

LIPID METABOLISM IN ACHOLEPLASMA LAIDLAWII

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This thesis is based on the following publications, which will be referred to in the text by their roman numerals, and some unpublished observations presented in section C.

- I. Control of membrane polar lipid composition in Acholeplasma laidlawii A by the extent of saturated fatty acid synthesis.
A. Christiansson and Å. Wieslander (1980)
Biochim. Biophys. Acta 595, 189-199
- II. Membrane lipid metabolism in Acholeplasma laidlawii A EF22.
Influence of cholesterol and temperature shift-down on incorporation of fatty acids and synthesis of membrane lipid species.
A. Christiansson and Å. Wieslander (1978)
Eur. J. Biochem. 85, 65-76
- III. Fractionation of membranes from Acholeplasma laidlawii A on basis of their surface properties by partition in two-polymer aqueous phase systems.
Å. Wieslander, A. Christiansson, H. Walter and C. Weibull (1979)
Biochim. Biophys. Acta 550, 1-15
- IV. Lipid bilayer stability in membranes. Regulation of lipid composition in Acholeplasma laidlawii as governed by molecular shape.
Å. Wieslander, A. Christiansson, L. Rilfors and G. Lindblom (1980)
Biochemistry 19, 3650-3655
- V. Effects of anesthetics on water permeability and lipid metabolism in Acholeplasma laidlawii membranes.
A. Christiansson, H. Gutman, Å. Wieslander and G. Lindblom (1981)
Accepted for publication in Biochim. Biophys. Acta
- VI. Protein composition and extractability of lipid-modified membranes from Acholeplasma laidlawii.
K.-E. Johansson, C. Jägersten, A. Christiansson and Å. Wieslander (1981)
Submitted to Biochemistry

A. INTRODUCTION

Acholeplasma laidlawii belongs to the mycoplasmas, the smallest autonomously growing organisms known to exist. The minimal cell diameter is approximately $0.3\ \mu\text{m}$ (1). In contrast to other bacteria, the mycoplasmas lack a cell wall, and the cell membrane constitutes the only barrier towards the environment. Due to the lack of cell wall and intracellular membranes, it is very easy to isolate the cell membrane in a pure form by osmotic lysis.

A. laidlawii was originally isolated from sewage (2), but occurs in a variety of domesticated animals as well as in man, and is a common contaminant in animal cell cultures (3). It has also been isolated from plants, and can multiply in insects (3). Undoubtedly, this variability in habitat must be reflected in an ability to adapt the cell membrane to the conditions prevailing in the different milieus.

The small size of the mycoplasmas is accompanied by a small genome (4) leading to a limited biosynthetic capacity. A. laidlawii depends on the growth medium for supply of sugars, most amino acids and nucleotides and also for unsaturated fatty acids (5). Saturated fatty acids are also taken up when present, as well as fatty acids with other structures and cholesterol. This makes A. laidlawii very suitable for membrane studies, since the membrane fatty acyl chain composition can be varied in a controllable way by the exogenous supply.

The membrane of A. laidlawii contains lipid and protein in approximately equal amounts on a weight basis. The lipids are organized in a bilayer (6), which can undergo a temperature-dependent gel to liquid crystalline phase transition (7) in a temperature interval which depends on the acyl chain composition (8). About 140 different polypeptides have been found in the Acholeplasma membrane (9), most of which are integral membrane proteins. A majority of the proteins are facing the cytoplasmic side of the membrane (9,10). The molecular weights range from less than 30 000 to 200 000 daltons (11). The majority of the polar membrane lipids are glucolipids, mainly monoglucosyldiglyceride (MGDG) and diglucosyldiglyceride (DGDG). Phosphatidylglycerol (PG) is the major ionic lipid species, in some strains

accompanied by diphosphatidylglycerol (DPG). Furthermore, glycerophosphoryl-derivatives of MGDG and DGDG, i.e. GPMGDG and GPDGDG, and an apolar monoglucolipid X (12) are found. Neutral lipids include mono- and diglycerides, found in small amounts, and carotenoids. Cholesterol, though not needed for growth, is taken up from the medium when present (13). Other membrane components are the lipopolysaccharides (14) and the hexoseamine polymers (15), which are not prominent on a molar basis. Furthermore, different ions, especially divalent cations like Mg^{2+} , may be firmly bound to the membrane (16).

Acholeplasma laidlawii has played an important role in the research concerning many fundamental aspects of membrane structure. Investigations of the organization of lipids and proteins in a biological membrane, and of the importance of the physical state of the membrane lipids for membrane permeability, protein disposition and function have been made with this organism (reviewed in 17 and 18). In these studies the advantage of incorporating different fatty acids into the membrane has been exploited. Much knowledge has accumulated in this field, but despite these studies relatively little (cf Escherichia coli (19)) is known about the lipid biosynthetic pathways in A. laidlawii and the factors governing how the different lipids are synthesized.

Fatty acid synthesis in A. laidlawii occurs in the cytoplasm via the malonyl-CoA pathway, (20) using acetate as a precursor (21). The products are saturated fatty acids only, since A. laidlawii lacks the enzyme necessary for the synthesis of unsaturated fatty acids (20). A. laidlawii can incorporate exogenous fatty acids and elongate them to some extent (22,23,24). This elongation system seems to have a broad specificity with respect to the chemical structure of the fatty acid (24). Incorporation of exogenous fatty acids leads to a decrease in the endogenous synthesis (25,26). Carotenoids can be synthesized de novo from acetate (27). Acholeplasma derives its energy from fermentation of glucose via the glycolysis (5). The organism is capable of de novo synthesis of its polar lipids; probably starting with sn-glycerol-3-phosphate, obtained from glyceraldehyde-3-phosphate in the glycolysis (13). The pathways leading to the polar lipids thus do not seem to be different from those found in other

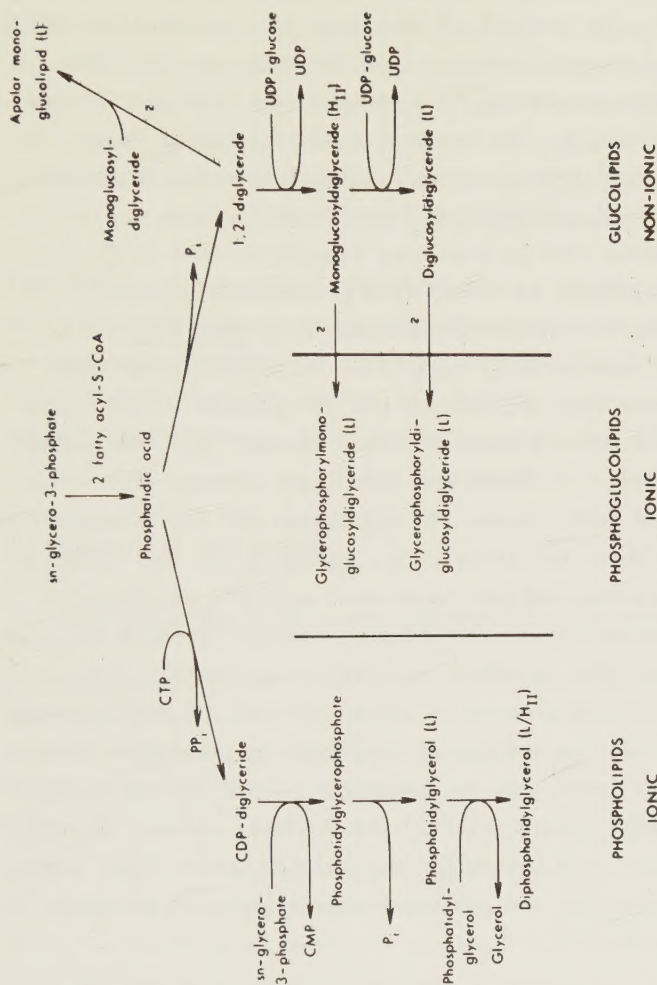


Figure 1: Tentative pathways for membrane polar lipid biosynthesis in *Escherichia coli* strain A. Abbreviations used: CMP, cytidine 5'-monophosphate; CDP, cytidine 5'-diphosphate; CTP, cytidine 5'-triphosphate; UDP, uridine 5'-diphosphate; P_i, inorganic orthophosphate; PP_i, inorganic pyrophosphate; CoA, coenzyme A; L, lamellar phase structure; H_{II}, reversed hexagonal phase structure; ?, suggested pathway.

bacteria, fig. 1 (13). Unsaturated fatty acids are preferentially incorporated in the sn-2-position of glycerol-3-phosphate and saturated fatty acids in position number 1 (28) by the action of one or more acyltransferases, situated in the membrane (29). Fatty acids with other chemical structures are also incorporated and their positional distribution seems to depend on their physical properties (30). The product of this reaction, phosphatidic acid, can be isolated in small amounts from the membrane (31). The formation of CDP-diglyceride from phosphatidic acid and CTP has been demonstrated (13). The enzymes involved in polar lipid synthesis all seem to be integral membrane proteins. Phosphatidylglycerol is probable synthesized from CDP-diglyceride and glycerol-phosphate (13) in a pathway similar to that in E. coli. Diphosphatidylglycerol is formed from phosphatidylglycerol. Although the exact mechanism is unknown in A. laidlawii, it is presumably the same as in E. coli (13). The synthesis of MGDG and DGDG proceeds from diglyceride and UDP-glucose in two steps catalyzed by one or more membrane-bound enzymes (32). The mechanisms for synthesis of GPMGDG and GPDGDG are unknown (13). Thus the synthesis of fatty acids, its regulation and the incorporation of fatty acids into the lipids of A. laidlawii are well investigated, but few investigations have dealt with the variation in polar lipid composition. Nevertheless, large differences in lipid composition have been reported, see references in (12). For A. laidlawii strain B changes in the molar ratio of MGDG and DGDG (33) and variations in phospholipid composition (34) have been observed during growth, but no systematic investigations were made. A. laidlawii strain A (EF22) was found to change its polar lipid composition in a systematic way related to the fatty acyl chain composition (12) and similar observations were recently made for strain B (35).

The organization of lipids and proteins in a biological membrane constitutes a complicated assembly designed to fulfill many functional demands. The bacterial cell membrane must function as a barrier between the cytoplasm and the surrounding medium, be a matrix for the membrane-bound proteins (enzymes), provide selective transport functions for nutrients and waste products, take part in the generation of an electrochemical potential

across the membrane, which is necessary as an energy buffer for the cell processes, etc. As regards the barrier function one single lipid species e.g. PG could very well form a bilayer impermeable for hydrophilic molecules. However, biological membranes contain many lipid species with different polar head groups. The reasons for this have been unknown, particularly since very few membrane bound enzymes have a demand for specific lipid species for their function (36). Recently Israelachvili et al. put forward a theory dealing with the assembly of lipid and protein molecules into a bilayer, reviewed in (37). This theory is based on both thermodynamic and packing considerations, i.e. on the shape of the lipid and protein molecules.

This thesis is concerned with observations on how fatty acyl chain and polar lipid head group composition are regulated in A. laidlawii (papers I, II and III) and provides a theory for why this regulation exists (paper IV). Finally, some observations supporting the theory is presented (papers V and VI).

B. SUMMARY OF THE PAPERS

1. Incorporation of fatty acyl chains into the membrane lipids.

A. laidlawii can incorporate either endogenously synthesized or exogenously supplied fatty acids into the membrane lipids (see above). In order to control the incorporation, it is important that the growth medium components are thoroughly lipid depleted (38), so that the incorporation of specifically supplied exogenous fatty acids will be as large as possible. However, there will still be a biosynthetic background of endogenous fatty acids (11). It was observed early in our laboratory that A. laidlawii A (EF22) had an unusually low level of endogenous synthesis compared with other strains, and that virtually no endogenous synthesis was present after 12 hours of growth (12). In paper I it was shown that the low level of fatty acid synthesis is due to a lack of pantetheine in the growth medium. Pantetheine is a precursor for coenzyme A and acyl carrier protein, which are needed in fatty acid biosynthesis (39). Addition of pantetheine resulted in a rapidly increased saturated fatty acid synthesis

and the proportion of endogenously synthesized acyl chains increased in the membrane lipids. Thus, at a given temperature, incorporation is dependent upon the relative proportions of fatty acids from endogenous and exogenous sources. Two other strains of A. laidlawii, B(ju) and B(JA1), had a similar low level of endogenous synthesis in the absence of pantetheine, showing that the results are not caused by any specific property of strain A (EF22), but is a consequence of the procedure for growth medium preparation. When A. laidlawii was grown in pantetheine-containing medium supplemented with oleic acid (an unsaturated fatty acid, needed for growth) and palmitic acid (a saturated fatty acid), the endogenous synthesis was suppressed. These results showed that using the lipid depleted medium lacking pantetheine, the endogenous fatty acid synthesis is very low. The use of radioactively labelled fatty acids to quantify acyl chain and polar lipid composition, is hence justified.

In paper II and III only exogenous fatty acids were used, either oleic acid alone, or a mixture of oleic and palmitic acids. Under the conditions used, more than 95% of total acyl chains were of exogenous origin at the end of the growth period (12).

In the experiments presented in paper III A. laidlawii was grown in media containing palmitic and oleic acids in different proportions. An increase in one of the fatty acids in the medium was paralleled by a similar increase in the proportion of the same acyl chain in the lipids (Table I and III in paper III), emphasizing the ease with which the fatty acyl chain composition can be manipulated. This indicates that under these conditions the properties of the acyltransferases per se are not the primary determinants of acyl chain composition.

In paper II cells were grown for 12 hours at 37°C. At that time the culture was divided, and half of the culture was shifted to 17°C while the other half remained at 37°C. The temperature shift resulted in an increase in the level of oleoyl chains in the lipids of cells grown with an equimolar mixture of palmitic and oleic acids. The uptake of oleic acid was even larger if cholesterol was present in the medium. Both a temperature decrease and the presence of cholesterol is known to increase

the molecular order of the membrane lipids (11). A. laidlawii thus possesses a "homeoviscous" adaptation mechanism sensing the physical state of the membrane lipids and compensating the increase in molecular order by an increased uptake of unsaturated fatty acids. The same effect of temperature on fatty acid uptake has been observed by others (40,41). The results are in accordance with the observations of Melchior and Steim that the uptake of saturated and unsaturated fatty acids by a bilayer is determined by the physical state of the bilayer itself (42).

The first product of the pathways leading to polar lipids is phosphatidic acid, from which all polar lipid species originate. Despite this, a unique fatty acyl chain composition was found for all lipid species. Phosphatidylglycerol was the most unsaturated species, whereas MGDG and glucolipid X were the most saturated ones (paper II).

2. Synthesis of polar lipid species.

The incorporation of different fatty acids results in drastic changes in the polar lipid composition of A. laidlawii membranes. In paper I the stimulation of endogenous saturated fatty acid synthesis, by addition of different amounts of pantetheine to the growth medium, was accompanied by a linear increase in the molar ratio between MGDG and DGDG with increasing amounts of saturated acyl chains in the membranes (fig. 2 in paper I). Furthermore, there was a concomitant decrease in the relative amount of ionic lipids (PG, GPMGDG, GPDGDG), i.e. the amounts of MGDG and DGDG increased. Another characteristic feature was the increase in the amount of glucolipid X when the saturated acyl chain content increased. This lipid was always highly saturated. These changes indicate a sensitive regulation system, changing the polar lipid composition upon changes in the hydrophobic parts of the bilayer. A sudden addition of pantetheine during growth resulted in a rapid synthesis of saturated fatty acids and an increased MGDG/DGDG ratio (fig. 4 in paper I).

Paper II describes the effects of two other factors affecting lipid composition. Since several different fatty acids affected the composition in a manner that tentatively could be correlated with their physical properties (12), a coupling between lipid synthesis and membrane molecular order was suspected. The effects of a temperature decrease and of cholesterol incorporation was therefore investigated. During growth at 37°C a culture supplemented with equimolar amounts of palmitic and oleic acids progressively incorporated more oleic acid (table 2A, paper II) and the MGDG/DGDG ratio decreased (fig. 5A, paper II), cf. above. After a shift to 17°C oleic acid incorporation increased and the MGDG/DGDG ratio increased as well (fig. 5A). The first lipid to be synthesized after the lag, caused by the shift, was MGDG. Simultaneously, the relative amount of ionic lipids decreased.

A temperature shift in a culture supplemented with oleic acid only, also resulted in an increase in the MGDG/DGDG ratio (fig. 5B, paper II). This shows that not only changes in acyl chain composition, but also a change in temperature per se affects this ratio. It should be noted that a reversed shift, from 26°C to 37°C, leads to a very rapid decrease in the MGDG/DGDG ratio (A. Christiansson, unpublished observation). When cholesterol was present in the growth medium the lipid metabolism was also affected, but the details in the regulation pattern were somewhat different from that without cholesterol (fig. 2, 5, paper II). Since the different factors, i.e. fatty acid supplementation, temperature and cholesterol, resulted in similar responses in lipid metabolism, it seemed that the apparent changes in the molecular order of the membrane lipids was an important factor governing polar lipid synthesis.

In paper III membranes from cells grown with different palmitic/oleic acid ratios in the medium were analyzed by counter-current distribution in a two-polymer aqueous phase system. This method separates membranes on basis of their surface properties (43,44). Using an appropriate polymer system, containing e.g. dextran and polyethylene glycol, very subtle differences in charge and hydrophobicity can be detected (43,44). It was found that the lateral organization of lipids in the Acholeplasma membrane was not homogenous, since populations of membranes with different surface

properties were detected. Lipid analysis showed that the lipid composition probably was important for this heterogeneity.

The total polar lipid composition varied with the acyl chain content in the same manner as shown in paper I. Thus, the MGDG/DGDG ratio decreased with increasing amounts of oleoyl chains in the lipids, the amount of glucolipid X decreased and that of the ionic lipids increased (table I and III, paper III). Assuming that molecular order is an important regulator of lipid composition, it might be expected that the presence of cholesterol, in a membrane containing oleoyl chains only, would make the membrane more ordered, and thus cause an increased MGDG/DGDG ratio (cf. temperature). However, the ratio decreased even further, and the amount of ionic lipids increased (table III, paper III). This also held for membranes from cells grown with cholesterol and other unsaturated fatty acids (18:2c, 18:3c) (A. Christiansson, unpublished). Thus molecular order seems not to be the cause of regulation of polar lipids. A similar conclusion was made recently for A. laidlawii B (35). In section C a theory is presented that accounts for this regulation.

3. Lipolytic enzymes and turnover.

The regulation of lipid composition in A. laidlawii has so far only been considered in terms of de novo synthesis of lipids. However, it might be possible that part of the variations were due to enzymatic breakdown and turnover of existing lipids. Some evidence for this is presented in paper II. PG can rapidly be converted to DPG and equally rapid be reconverted to PG, implying the presence of lipolytic enzymes capable of this degradation. Furthermore, a degradation or turnover of PG could be detected in some instances, since the oleoyl chain content in PG increased, despite a total decrease in PG amounts (fig. 2A and table 2A, paper II). Similar observations were made for MGDG. However, no results indicated that turnover played a major role in the lipid regulation mechanism. No lipolytic activity was detected when isolated cell membranes were incubated (paper II), but incubation of living cells with isolated membrane lipids from A. laidlawii indicated that all the polar lipids can be degraded

by endogenous lipolytic enzymes (45). The significance of these lipolytic activities during normal growth remains unknown, but it can be speculated that they fulfill a role during rapid adaptations to new environmental conditions. A lysophospholipase activity has been found in *A. laidlawii* B (46) but no lipid turnover has been demonstrated during exponential growth (47,48).

4. A physical basis for regulation of lipid composition.

A theory that accounts for the regulation of lipids in a membrane must necessarily take into account the physical properties of the lipid and protein molecules and how they interact. Lipids are composed of a hydrophobic part, the acyl chains, and a hydrophilic part, the polar head group. When lipid molecules are dispersed in water, the acyl chains will be eliminated from the water phase, due to the hydrophobic effect (49), and they pack together. The polar head groups will be oriented towards the water phase. Due to size, charge or degree of hydration, repulsive forces will act between the head groups, tending to keep them apart. Entropy always favours small aggregates, but larger ones often form in order to minimize the total free interaction energy (attractive and repulsive forces) of the system. Thus depending on the type of lipid molecule, aggregates with different symmetries may form, e.g. spheres, cylinders or lamellae. The assembly of lipids into bilayers and other aggregates has been described by Israelachvili in a theory that links thermodynamics, interaction free energy and molecular shape (50,51). The formation of different aggregates can be explained qualitatively by regarding the lipids as building bricks with different shapes. The shape of a brick will be determined by the length (l) and volume (v) of the hydrocarbon chains and the optimal surface area (a) of the molecule at the hydrocarbon-water interface, cf. fig. 2 in paper IV. The optimal surface area (a) depends on the chemical structure, the degree of hydration and the charge of the head group. The parameter (l) depends on the number of carbon atoms in the acyl chains, but also on the degree of unsaturation. The shape of the hydrophobic part will furthermore depend on the temperature. A temperature

increase will increase the thermal motions of the acyl chains leading to a shortened and broadened hydrophobic part of the molecule (fig. 2, paper IV).

Most lipids isolated from biological membranes form a lamellar phase when dispersed in water. However, almost every membrane contains lipids forming other types of phase structures, like a reversed hexagonal (H_{II}) phase (37). MGDG from A. laidlawii is an example of this (52) and can be visualized as a cone (Δ with polar head at top). The other membrane lipids isolated from A. laidlawii form a lamellar phase (52) and can be regarded as rod-shaped. An intact bilayer is necessary for the function of a cell. Thus, in A. laidlawii lipids with different molecular shapes must be able to pack together so that a bilayer is formed.

In paper IV the observed lipid regulation presented in papers I-III is examined in terms of the packing properties of the lipids. It is proposed that an optimal packing of the lipids is necessary for membrane function, and that the cell compensates for packing changes to reestablish this optimum. For example an increased unsaturated acyl chain content in the lipids, will lead to a more cone-shaped geometry of all lipids. Still MGDG will be the most cone-shaped lipid. MGDG and the other lipids may then be too cone-shaped to be accommodated together in a bilayer. The ratio MGDG/DGDG is therefore lowered to restore membrane stability. On the other hand, a temperature-shift from 37 to 17°C gives the hydrophobic part of the lipids a less cone-shaped appearance. In order to maintain an optimal packing stability, the amount of MGDG (and its degree of acyl chain unsaturation) is increased. Furthermore, an increased content of unsaturated acyl chains yields a decrease in the surface charge density, which leads to an increased synthesis of ionic lipids. This may be due, at least partly, to a compensation for changes in lateral lipid packing. Cholesterol, like MGDG, is a cone-shaped molecule (53). Cholesterol and MGDG are not mutually compatible in large amounts in a bilayer. Upon incorporation of cholesterol the MGDG/DGDG ratio is therefore decreased. Furthermore, the presence of cholesterol dilutes the surface charges and A. laidlawii compensates for this by an increase in the ionic lipid fraction (table I in paper IV).

A suggestion as to why biological membranes contain different kinds of lipid molecules is finally made. Since membrane proteins have different shapes, different kinds of lipids may be necessary to pack the proteins into the bilayer in a stable way, and for a proper function of the proteins (36,37).

5. Supporting observations.

If the factors outlined above are important for regulation of the lipid composition in A. laidlawii membranes, then the effect of other disturbances should be predictable, provided that the effect on the packing of membrane components can be foreseen.

Anesthetics comprise a large group of chemically unrelated molecules, whose anesthetic potencies seems to be correlated with their lipid solubilities (54). These molecules can therefore be expected to perturb the structure of membranes. Two anesthetics, diethylether and tetracaine were tested. In experiments analogous to the temperature shift experiments in paper II, they were added to growing cells. Since these anesthetics are lipophilic they penetrate into the hydrocarbon interior of the membrane and increase the hydrophobic volume (v). For packing reasons, the MGDG/DGDG ratio can be expected to decrease, which was also observed (fig. 3 in paper V). The anesthetics were used in concentrations commonly used for anesthesia, which gives a membrane concentration of one anesthetic molecules per 12-15 lipid molecules. This results in a very small change in surface charge density and no regulation in ionic lipid amounts was observed in the diethylether supplemented culture. In the culture supplemented with tetracaine the amount of ionic lipids increased, probably to compensate for the positive charge of tetracaine. Both the changes in glucolipids and ionic lipids were very small and no difference in the permeability of water over the bilayer after addition of anesthetics could be detected by NMR measurements. This indicates that the lipid regulatory system is very sensitive.

A theory for lipid regulation based on the physical properties of the lipids should be applicable not only to A. laidlawii A (EF22), used in the investigations hitherto, but also to other strains of A. laidlawii. It was found that strain B(me), obtained from Dr. R.N. McElhaney, Edmonton, Canada had the same low level of endogenous saturated fatty acid synthesis as strain A (EF22) in our growth medium, and responded in the same way, regarding the polar head group regulation upon pantetheine addition (A. Christiansson unpublished), cf. (35). Furthermore, strain B(ju) obtained from Dr. K.-E. Johansson, Uppsala, was adapted to our growth medium and grown with different fatty acid supplementations as in paper III. Growth in a medium with PPL0-serum instead of fatty acids was also investigated. Paper VI shows that the direction of changes in the MGDG/DGDG ratio and in the amount of ionic lipids were the same as in A. laidlawii A (EF22) indicating that the same regulation system is at work (table 2, paper VI). The combined effect of cholesterol and linoleic acid in the PPL0-serum supplemented culture gives a further decrease in the MGDG/DGDG ratio and in increased ionic fraction. The membranes from cells grown with different fatty acid supplements were extracted with the nonionic detergent Tween 20, which selectively extracts proteins and lipids (55), probably yielding micelles containing Tween 20, membrane lipids and proteins. The molecular shape of Tween 20 can be visualized as a cone (▽ with polar head at top), and Tween packs into micelles as predicted from the molecular shape (50). The action of detergents on biological membranes depends on the ratio of detergent to membrane components (56). The ratio employed here gives a disruption of the membrane and a partial extraction of membrane components. When the extracted material was compared with the membrane residue it was found that the ionic lipids and DGDG were enriched in the supernatant, whereas MGDG was enriched in the membrane residue (table 2 and 4, paper VI). This is logical in terms of molecular packing, since MGDG and Tween 20 cannot pack together in a micelle due to the high radius of curvature and MGDG is therefore left behind in the membrane residue. Furthermore, the extractabilities of several membrane proteins were found to be dependent on the lipid composition of the membranes, indicating the importance of the lipids for the embedding of the proteins in the membrane and for

their extractability with nonionic detergents. Particularly, a coupling between the extractability of protein D₁₂ and the amount of MGDG was found, which indicates a coupling between these two components in the membrane.

MGDG and DGDG constitutes the majority of the lipids in A. laidlawii, and MGDG is the only lipid isolated from this organism that forms a H_{II} phase. In vitro mixtures of MGDG and DGDG in different ratios and with different degrees of acyl chain unsaturation were found to form a lamellar or a cubic phase in a manner predictable from the theory of Israelachvili (57). Furthermore, the destabilizing effect of cholesterol on MGDG/DGDG mixtures was also demonstrated (58). All mixtures of lipids with ratios found in vivo formed bilayers, whereas non-physiological mixtures yielded cubic or hexagonal phases (59). The MGDG/DGDG ratio was higher in a mutant of A. laidlawii resistant to reactive complement lysis (60) than in the wild-type, and a correlation has been found between the glucolipid ratio and resistance against infection with mycoplasma virus MVL51 (61).

C. A METABOLIC MODEL FOR REGULATION OF LIPID COMPOSITION IN ACHOLEPLASMA LAIDLAWII

Although the basic principles governing the regulation of lipid composition has been explained on a physicochemical basis, the actual regulation must be effected by the lipid-synthesizing enzymes. Furthermore, the variation in the amounts of all the lipid species must be taken into account. In this chapter a tentative model for this regulation is given. It is compatible with the major observations in the papers of this thesis, though speculative in some details.

Table 1 shows the lipid and acyl chain composition of membranes from Acholeplasma laidlawii B(ju) grown with different fatty acid supplements. Also the minor lipids are shown. Fig. 1 shows the suggested pathways for polar lipid synthesis, based on the knowledge in section A. The first step in lipid synthesis is the acylation of sn-glycerol-3-phosphate with two fatty acyl-CoA molecules to yield phosphatidic acid. In general, fatty acids

Table 1. Lipid composition in membranes from *Acholeplasma laidlawii* B(ju) grown with different amounts of saturated and unsaturated fatty acids (24 hours, 30°C). The lipid composition was determined by liquid scintillation counting.

Lipid	PPL0 serum fraction ^{a)}		Supplementation to the growth medium palmitic/oleic acid (μM/μM)							
			0/150		30/120		75/75		120/50	
	mol%	% UFA ^{b)} ND ^{c)}	mol%	% UFA	mol%	% UFA	mol%	% UFA	mol%	% UFA
CPDGCG	4.3	"	7.1	ND	7.1	83.3	4.2	65.4	4.2	52.4
CPMGCG	9.0	"	1.5	"	0.5	82.0	2.6	61.0	1.2	36.5
PC	34.6	"	27.4	"	32.9	88.5	27.8	59.9	17.3	34.5
DGCG	40.1	"	47.2	"	42.1	82.6	42.8	61.0	37.3	45.5
MGCG	11.4	"	16.6	"	17.2	80.6	21.8	43.3	23.2	16.6
GLX	0.6	"	0.1	"	0.1	71.4	0.8	21.6	12.7	13.8
Neutral lipids	0.1	"	0.1	"	0.2	70.4	0.1	32.3	4.2	4.8
Average % UFA	25% 18:2c ^{d)} 60% 18:1c		95.0 ^{d)}		84.3		56.7		29.7	
Molar ratio MGCG/DGCG	0.28		0.35		0.41		0.51		6.62	
Total % ionic lip.	47.9		36.0		40.5		34.6		22.7	
Total % ^{e)} CP-der.	13.3		8.6		7.6		6.8		5.4	
Total % lipids derived from MGCG	65.3		72.5		66.9		72.1		78.5	

a) PPL0 serum fraction (Difco laboratories), commercial serum for mycoplasmas acting here as a source of oleic (18:1c), linoleic (18:2c) acid and cholesterol. Palmitic acid was not added.

b) % UFA: mol% unsaturated acyl chains in the individual lipids.

c) ND not determined

d) determined by gas liquid chromatography

e) CP-der.: Total % of CPMGCG and CPDGCG

with low melting points are acylated in position 2 whereas high-melting fatty acids are acylated in position 1. In *E. coli* two enzymes, acting sequentially, are responsible for these reactions (62), but the situation in *A. laidlawii* is not known. However, the positional selectivity is not absolute, as can be inferred from the data on acyl chain composition in table 1. Actually there is a linear relationship between the fatty acid composition of the medium and the acyl chain composition in the lipids (fig. 2A). Thus, despite the positional selectivity (28), the acyl-transferases accept fatty acids in proportion to their availability in the medium (cf. section B1). The acyl chain composition of the lipids is also affected by growth temperature and membrane cholesterol content (paper II). The two latter observations can be explained by regarding the lipid bilayer as a temperature-programmed sink, taking up proportionally more unsaturated fatty acids when the membrane is in a more ordered state, and more saturated fatty acids when less ordered, as

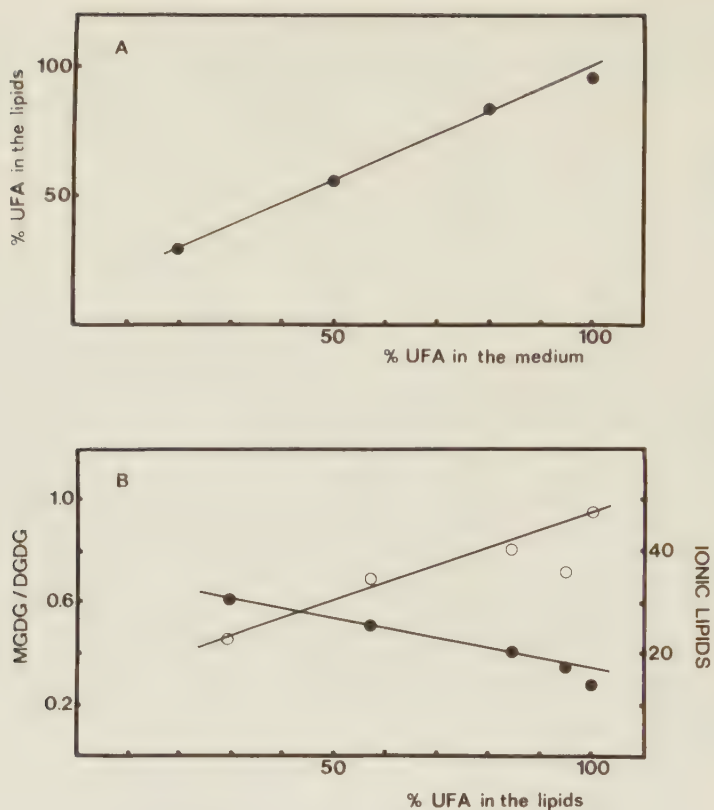


Figure 2: Lipid composition of *A. laidlawii* 8(ju) grown with different fatty acid supplements. A summary of the data in Table 1.
 A. The dependence of the lipid acyl chain composition on the fatty acid composition in the medium. UFA: unsaturated acyl chains.
 B. The MGDG/DG DG ratio and the percentage of ionic lipids in membranes with different acyl chain compositions. The degree of acyl chain unsaturation in the PPLD sample (Table 1) was set equivalent to 100% of oleic acid.

suggested by Melchior and Steim (63). These authors propose that the uptake of different fatty acids by a membrane is a consequence of the thermodynamic state of the bilayer and not primarily due to the selectivity of the acyltransferases.

From phosphatidic acid (PA) two major pathways diverge: the pathway leading to PG and the one leading to the glucolipids. An interesting observation is that PG in general has a higher content of unsaturated acyl chains than the average. In particular, MGDG and glucolipid X are the most saturated species (table 1).

This implies that phosphatidic acid with different degrees of acyl chain unsaturation is selectively chosen by the pathways leading to PG and MGDG. In vitro assays for the synthesis of MGDG and DGDG was possible with 1,2-diglyceride derived from A. laidlawii lipids but less effective with egg yolk diglyceride (32) substantiating a selectivity on basis of acyl chain composition. Furthermore, in vitro assays for CDP-diglyceride synthesis in E. coli was not possible using 1,2-dipalmitoyl phosphatidic acid, but worked with more unsaturated species (64). A selectivity for, different phosphatidic acid species (or their physical properties) may thus be one mechanism governing the relative amounts of PG and glucolipids. The observation of different "pools" of phosphatidic acid in E. coli (65) is also compatible with this model, since phosphatidyl-ethanolamine (PE) (which is synthesized by one of the two lipid biosynthetic pathways in E. coli) was always more saturated than PG and DPG (synthesized in the other pathway) (66). It is interesting to note that MGDG and PE, both of which can form a H_{II} phase (52,67), are the least unsaturated major lipid species in E. coli and A. laidlawii respectively. Another possible factor governing the relative synthesis of PG and glucolipids may be the membrane surface charge. The lipid biosynthetic enzymes are localized in the membrane. One of the substrates for phosphatidylglycerophosphate synthetase (the first enzyme in the PG pathway), i.e. glycerol-phosphate, is water soluble and negatively charged, and must approach the membrane to reach the enzyme. The membrane has a negative surface charge (68) due to the presence of ionic lipids, and a negative Gouy-Chapman potential exists, reaching out from the bilayer into the fluid (69). This potential makes it more difficult for negatively charged molecules than for positively charged ones to approach the membrane. If the surface charge density is diluted, e.g. by an increased unsaturated acyl chain content of the lipids, the magnitude of this potential will decrease, making glycerol-phosphate more available to the enzyme. This may lead to an increased synthesis of PG, cf. table 1 and fig. 2B. A similar mechanism has been proposed to regulate the activity of other membrane-bound enzymes (70). An increase in the synthesis of PG will lead to a concomitant decrease in the total output of glucolipids, cf. table 1. This indicates that the level of phosphatidic

acid (PA) is not the main target for the regulation mechanism.

In A. laidlawii A (EF22) and B(ju) usually very little DPG is synthesized, but the potential capacity for rapid synthesis exists (fig. 2C in paper II, where PG rapidly is converted to DPG). It is noteworthy that DPG can form a H_{II} phase in the presence of divalent cations (71), and the conversion between PG and DPG may be a way to adjust packing constraints in the membrane. The actual enzymatic mechanisms governing this reaction, as well as the MGDG/DGDG conversion, which obviously somehow is governed by the packing constraints, is unknown. However, it is well known that the activity of membrane-bound enzymes can be affected by the lipids and requirements for lipids with specific structures may govern enzyme activity (36). It can be observed that when unsaturated 1,2-diglycerides are accepted by the glucolipid pathway, a smaller MGDG/DGDG ratio is obtained (fig. 2B), i.e. MGDG is converted further to DGDG. The acyl chains of DGDG are always more unsaturated than those of MGDG and the difference is larger the more saturated acyl chains that are present totally in the membrane (table 1). Fig. 2B illustrates the linear relationships between acyl chain unsaturation, the MGDG/DGDG ratio and the percentage of ionic lipids, respectively. This also holds for strain A (EF22), cf. fig. 2 in paper I. The levels of the MGDG/DGDG ratios and the amount of ionic lipids are dependent upon the growth temperature and the strain used but the trends are similar (A. Christiansson, unpublished).

The glycerophosphoryl derivatives of MGDG and DGDG, i.e. GPMGDG and GPDGDG are most probably synthesized from MGDG and DGDG, though the mechanisms are unknown (13). The GP-derivatives are more unsaturated than the corresponding glucolipids but their acyl chain compositions seem to reflect their origin from the two glucolipids. From the point of packing properties, it seems logical that the charged derivatives can be allowed to have a higher degree of unsaturated acyl chains than their uncharged parent molecules. The regulation of the amounts of the GP-derivatives (GPDGDG and GPMGDG) do not seem to simply reflect the total glucolipid amounts. It seems rather to parallell the amounts of PG (table 1), suggesting that surface charge regulation may be active (fig. 2B).

Finally, both the exact structure and the biosynthetic pathway for glucolipid X (GLX) is unknown. This lipid contains glucose, fatty acids and glycerol in a molar ratio of 1:4:2 and has been suggested to consist of two diglyceride molecules linked to glucose (1,12). This lipid is synthesized in large amounts only in the presence of high concentrations of saturated fatty acids in the medium, and is usually the most saturated polar lipid species (table 1). It is possible that this lipid acts as a scavenger for extremely saturated diglycerides, not readily acceptable by the MGDG-synthesizing enzyme. This supports the previous notion that the amount of lipids is not regulated at the PA level.

D. CONCLUDING REMARKS AND DISCUSSION

The results shown above indicates a sensitive, self-regulating system aiming at homeostasis as regards lipid packing. Fatty acid uptake can be envisaged to be dependent on the physical state of the membrane bilayer itself, the balance between ionic and nonionic lipids to be dependent on membrane surface charge and the glucolipid composition to depend on the molecular geometry and the phase structure of these lipids.

An interesting parallel as regards fatty acylchain regulation can be made with the Tetrahymena cell, where the action of a fatty acid desaturase seems to depend on the physical properties of the membranes and regulate the output of unsaturated fatty acids accordingly, reviewed in (63).

The central reasoning concerning lipid packing properties has, in addition to Acholeplasma lipids (58,59), been investigated in many other model membrane systems consisting of lipids forming lamellar and nonlamellar phases (72). It has been shown that depending on the proportion of different lipids, the pH and the presence of ions, lamellar or nonlamellar phases can be formed. The results can be rationalized in terms of molecular geometry (72). Sphingomyelin was shown to stabilize phosphatidylethanolamine and cholesterol in a bilayer conformation, which would not be formed otherwise. The level of sphingomyelin in the membranes

of the arterial walls is elevated in the early stages of arteriosclerosis, and it has been suggested that this is a regulation to maintain a stable bilayer upon cholesterol incorporation (73).

Although this investigation has dealt only with the lipid regulation mechanisms in Acholeplasma laidlawii, the principles can nevertheless be expected to hold for other organisms as well. To my knowledge no other examples of regulation, except (73), have been interpreted in terms of molecular shape, but several examples can be given indicating the plausibility of the proposal. Changes in the degree of acyl chain unsaturation and in the amounts of monogalactosyl diacylglycerol (structurally similar to MGDG) was shown to play a central role in temperature adaptation in the blue-green algae Anabaena variabilis (74). Mycoplasma mycoides normally has a demand for cholesterol, but can be adapted to grow in a medium with low cholesterol concentration. When decreasing the amount of cholesterol in the membrane, there is a concomitant increase in the level of diglycerides (75), which have a molecular shape similar to that of cholesterol. In E. coli phosphatidylethanolamine (PE) is the dominant lipid, and can potentially form a H_{II} phase (67,76). The other major lipids are PG and DPG. Upon addition of a bulky, lipophilic morphine analogue to an E. coli culture, the amount of PE was strongly reduced in the membrane (77). Furthermore, mutants of E. coli resistant to organic solvents (also affecting the hydrophobic interior of the bilayer) were shown to have a lower PE content than the wildtype (78). In Pseudomonas halosaccharolytica the content of PE was reduced upon a temperature increase (79). An example of a linked fatty acyl chain and polar head group metabolism can be found in murine fibroblasts (80). A surface charge regulation is probably active in E. coli (19) and Neurospora crassa (81), where a fairly constant proportion of ionic lipids is maintained, regardless of different mutations in the polar lipid synthesis.

A biological membrane contains proteins in addition to lipids, and it was suggested that an important reason for the presence of lipids with different molecular shapes is to minimize packing constraints around the irregular surfaces of proteins (36,37).

Indeed, in model systems the introduction of proteins have been shown to stabilize or destabilize the bilayer structure, depending on the precise nature of the protein and the lipids (82,83). The presence of lipids with tendencies to form a H_{II} phase has also been suggested to be important for processes like membrane fusion, transbilayer lipid movements, exo- and endocytosis (reviewed in 72) and membrane transport of ions (84).

In conclusion, the model for regulation of lipid composition in A. laidlawii has been shown to describe in a qualitative way the observed changes in lipid composition. For a more detailed description it will most certainly be necessary to take into account the lateral distribution of lipids and proteins (85,86,87) as well as lipid and protein asymmetry (37,88,89). Other factors, like the electrochemical gradient generated over the membrane by the cell itself, also seems to affect lipid (90) and protein organization (91), and thus probably lipid metabolism.

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G. APPENDIX: PAPERS I-VI

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CONTROL OF MEMBRANE POLAR LIPID COMPOSITION IN *ACHOLEPLASMA LAIDLAWII* A BY THE EXTENT OF SATURATED FATTY ACID SYNTHESIS

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Key words: Fatty acid synthesis; Polar head group; Fluidity; Surface charge density; Lipid composition; (*A. laidlawii*)

Summary

The low level of endogenous fatty acid synthesis in *Acholeplasma laidlawii* A strain EF22 was found to be caused by a deficiency of pantetheine in the lipid-depleted growth medium. By supplementing the oleic acid-containing medium with increasing concentrations of pantetheine, saturated fatty acid synthesis was stimulated (having an apparent K_m of 5 μ M for pantetheine) and the incorporation of endogenously synthesized fatty acids in membrane lipids increased markedly. Furthermore, carotenoid biosynthesis was stimulated. Exogenous palmitic acid was found to inhibit partially the endogenous fatty acid synthesis. A gradual stimulation of fatty acid synthesis was accompanied by a linear increase in the molar proportion between the two dominating membrane glucolipids, monoglucosyldiacylglycerol and diglucosyldiacylglycerol. The total amount of charged membrane lipids decreased upon increasing the degree of fatty acid saturation. These regulations are discussed in terms of membrane stability, and influence of membrane molecular ordering and surface charge density on lipid polar head group synthesis.

Introduction

The mycoplasmas have played an important role in membrane molecular biology as tools for elucidating membrane structure and function [1,2]. Much is known about the importance of the physical properties of the fatty acids for the permeability, viscosity and phase transitions of the lipid bilayer [1]. However, very little is known about the function of the different polar head groups of the membrane lipids and the factors that regulate their synthesis.

In a previous investigation we found that the relative amounts of gluco- and phospholipids in the membrane of *Acholeplasma laidlawii* A strain EF 22 varied as a consequence of different fatty acid supplementation to the lipid-depleted but otherwise complex growth medium [3]. In this strain glucolipids comprise more than 50% (mol/mol) of the total membrane polar lipids [3]. The molar ratio between the two major glucolipids, monoglucosyldiacylglycerol and diglucosyldiacylglycerol, was low when the membrane lipids contained only an unsaturated fatty acid, whereas the presence of a saturated fatty acid increased this ratio. This was true for several fatty acids, fatty acid mixtures and cholesterol and it seemed that the physical properties of the fatty acids in the membrane were important for the balance between the glucolipids. This balance could thus possibly depend on the molecular ordering in the membrane. Further evidence in favour of this theory was obtained in temperature-shift experiments [4]. A high degree of molecular ordering resulted in a high monoglucosyldiacylglycerol/diglucosyldiacylglycerol ratio and a lower ordering yielded a lower ratio. The physical properties of monoglucosyldiacylglycerol and diglucosyldiacylglycerol are quite different [5]. Moreover, there was a variation in the degree of lipid fatty acid unsaturation with temperature, indicating that more than one regulatory mechanism was present [4].

When the growth medium is supplied with appropriate unsaturated fatty acids to sustain growth, *A. laidlawii* A strain EF22 has an unusually low level of endogenous saturated fatty acid synthesis compared to other *A. laidlawii* strains [3]. The reason for this has been assumed to be a block or deficiency in saturated fatty acid synthesis. If this synthetic activity could be enhanced, the increased saturated fatty acid content of the lipids, giving a higher molecular ordering, might be yet another means to control the glucolipid amounts in the cell membrane and to more closely examine the relationship between the saturated fatty acid content and the glucolipid ratio. This requires that the endogenously produced fatty acids are utilized in the same way in lipid synthesis as the exogenously incorporated ones. The resulting alteration in molecular ordering could be accomplished without invoking any form of exogenous influence on the organism, as was the case in the previous investigations.

In the present investigation we have therefore made efforts to stimulate the fatty acid biosynthetic machinery in *A. laidlawii*. The effects of pantetheine, a precursor for coenzyme A and acyl carrier protein, on lipid synthesis in *A. laidlawii* A strain EF22 are presented. The results show that the monoglucosyldiacylglycerol/diglucosyldiacylglycerol ratio can be altered as a result of the molecular ordering changes in the presence or absence of endogenous fatty acid synthesis. Furthermore, the amount of charged lipids are dependent upon the degree of fatty acid saturation.

Materials and Methods

Organisms and growth conditions. *A. laidlawii* A, strain EF22 was used in all experiments. Furthermore, *A. laidlawii* strain B(ju), obtained from Dr. K.-E. Johansson, Uppsala, and B strain JA1 from Dr. A. Liss, N.I.H., Bethesda, MD, were used in control experiments. The organisms were grown in a tryptose/bovine serum albumin medium [4] containing lipid-depleted tryptose [6] and

lipid-depleted bovine serum albumin (Cohn V) [6]. However, thallium acetate was not included in the medium. The original procedure for lipid depletion of tryptose was modified by performing the third lipid extraction with chloroform/methanol (2 : 1, v/v) overnight instead of for merely 2 h. The medium was supplemented with fatty acids in ethanolic solutions; 50 μ M oleic acid, alternatively 75 μ M oleic plus 75 μ M palmitic acid (Sigma Chemical Co). Stimulation of the endogenous fatty acid synthesis was achieved by adding pantetheine in concentrations from 0 to 40 μ M. In most experiments 12 μ M was used. The membrane lipids were labelled by adding 10 μ Ci/l of [14 C]oleic acid (59.7 Ci/mol) and in some experiments 10 μ Ci/l of [14 C]palmitic acid (57.9 Ci/mol), (The Radiochemical Centre, Amersham). To monitor the extent of fatty acid synthesis, 0.20 mCi/l of [3 H]acetate (100 Ci/mol, New England Nuclear Co.) was included.

The organisms were subcultured at 37°C in medium with each type of supplementation at least four times for adaptation. For the experiments, a 2% inoculum was grown until late logarithmic phase, i.e. 18 h, and the cells were then harvested. In shift experiments, a 12 h culture was divided in two parts, one of which was supplemented with 12 μ M of pantetheine while the other remained unsupplemented. Both cultures were incubated further and growth was estimated by absorbance measurements at 540 nm. Samples were withdrawn for membrane lipid and protein analysis at intervals. Cells were harvested by centrifugation at $32\,000 \times g$ for 10 min at 5°C in a Beckman J21 centrifuge and washed once in β -buffer [4]. Membranes were prepared, washed and freeze-dried according to [4].

Lipid analysis. Membranes were extracted and the lipid extracts were purified exactly as described before [4]. Parts of the extracts were taken to dryness and methylated [3]. Fatty acid composition was determined by gas-liquid chromatography [3]. Furthermore, the fatty acid composition of uninoculated growth medium was determined.

The individual lipid species were separated by thin-layer chromatography on silica gel H (Merck) plates (buffered with 1% (w/v) $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) in chloroform/methanol/water (65 : 25 : 4, v/v) for polar lipids, or on unbuffered plates in petroleum ether (b.p. 40–60°C)/diethyl ether/acetic acid (80 : 20 : 1, v/v) for neutral lipids. Lipids were visualized by iodine vapour. The lipid-containing zones were scraped off the plates and the lipids were eluted into scintillation vials as described [4]. After evaporation of the solvent, scintillation cocktail was added (Omnifluor, New England Nuclear Co.) and radioactivity was determined by liquid scintillation counting [4].

The amounts of unsaturated (oleic) acid relative to saturated fatty acids were determined by gas chromatography. By combination with the data from liquid scintillation counting, an estimation of the molar amounts of exogenously incorporated and endogenous synthesized fatty acids in the total lipid extracts and in the individual lipid species could be calculated. The ratio of unsaturated to saturated fatty acids in the lipids are almost the same with as well as without pantetheine (Table I). In the presence of exogenous oleic plus palmitic acids and pantetheine, the total amounts of endogenously synthesized fatty acids were estimated by assuming that the molar ratio of oleic/palmitic acids (exogenously incorporated) is the same as in cells grown without pantetheine.

Analytical methods. Protein was determined by the method of Lowry et al. [7] using bovine serum albumin (Cohn V) as a standard.

Carotenoids were tentatively identified by spectrophotometry wave-length scanning in hexane solutions.

The membrane protein patterns of the different cultures were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis [8].

Results

Endogenous saturated fatty acid synthesis in different A. laidlawii strains

A. laidlawii strain A has an absolute requirement for an unsaturated fatty acid, which it cannot synthesize itself [9], whereas the B strain can manage without unsaturated fatty acids [10]. Saturated fatty acids can, however, be synthesized by both strains. The requirement for unsaturated fatty acids was verified for our strain EF22 as before [3]. Addition of pantetheine to media containing saturated fatty acids only, did not make the growth medium able to sustain growth of *A. laidlawii* A (EF 22). Therefore, oleic acid was always included in the following experiments.

In order to assess the efficiency of the lipid extraction procedure for the growth medium components, residual fatty acids from uninoculated lipid-extracted medium containing 75 μ M oleic acid were analyzed by gas-liquid chromatography. Less than 4% (mol/mol) of the membrane fatty acids were of medium origin. When cells were grown in this medium, total lipid extracts from strain A (EF 22) contained 3.6% fatty acids other than oleic acid, strain B(ju) 4.0% and strain B(JA1) 17% other fatty acids. The JA1 polar membrane lipid composition differs from that of the other strains (Steinick, L.E., personal communication).

Fatty acid synthesis in *A. laidlawii* proceeds via the malonyl-CoA pathway, employing acetate as a precursor [11]. [3 H]Acetate was incorporated into fatty acids under these conditions, indicating that a low level of endogenous synthesis was present. We did not attempt to discriminate between the minimal amounts of fatty acids originating from the medium and endogenously produced fatty acids in the further experiments with *A. laidlawii* A (EF22). Therefore endogenous synthesis in the following results is actually a slight over-estimation of this capacity, since residual medium fatty acids are included.

All strains exhibited a lower degree of endogenous saturated fatty acid synthesis than reported earlier for *A. laidlawii* in lipid-depleted oleic acid-containing media [12,13]. The low level of endogenous synthesis is thus not an exclusive property of *A. laidlawii* A (EF22). The main reason for the diminished capacity of fatty acid synthesis seems to be a consequence of the procedure for medium preparation.

Regulation of saturated fatty acid synthesis by pantetheine and palmitic acid in A. laidlawii A (EF22)

The results obtained above indicate a deficiency in the growth medium of some component necessary for fatty acid synthesis. We have shown earlier that this was not due to a lack of acetate [3]. Pantetheine is known to be a biosynthetic precursor for the prosthetic groups of coenzyme A and acyl carrier protein, both of which are needed in fatty acid synthesis. Pantetheine stimulated

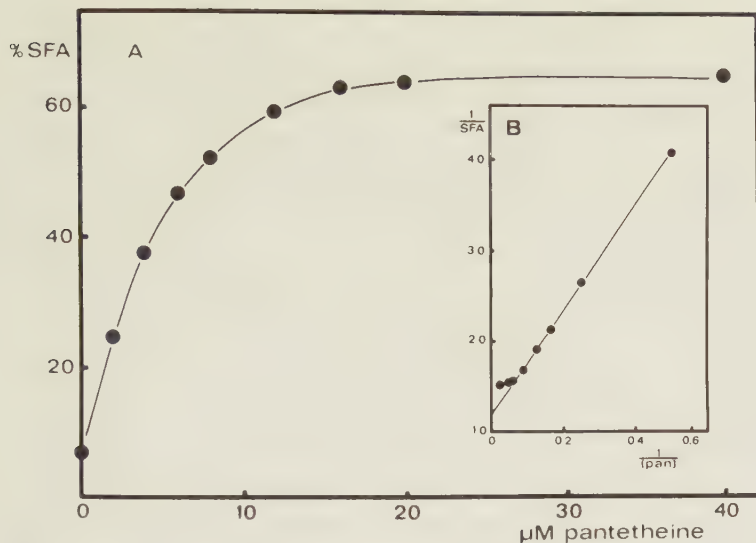


Fig. 1. Effect of pantetheine concentration on the extent of endogenous saturated fatty acid synthesis in *A. laidlawii* A (EF22). Cultures were grown at 37°C for 18 h in a lipid-depleted bovine serum albumin/tryptose medium supplemented with $50\ \mu\text{M}$ of oleic acid. Fatty acids were analyzed by gas-liquid chromatography. (A) Saturated fatty acid (SFA) content (mol/100 mol) of membrane lipids at different concentrations of pantetheine. (B) (Inset) Lineweaver-Burk plot of the data in (A).

acyl carrier protein activity in *A. laidlawii* A (oral strain) [14] when grown in a partly defined medium [15].

Fig. 1 shows the effect of increased pantetheine concentrations on the content of saturated fatty acids in the membrane lipids. Obviously lack of pantetheine limits fatty acid synthesis in the unsupplemented medium and an increase from approx. 5% to 65% (mol/mol) saturated fatty acids was observed upon pantetheine addition. Furthermore, cell yield increased by 75% as measured by absorbance at 540 nm. Usually $150\ \mu\text{M}$ of exogenous fatty acids are added to give optimal growth [3]. Consequently $50\ \mu\text{M}$ oleic acid will limit growth and a stimulation of fatty acid synthesis will increase growth. Fig. 1A indicates that the synthesis of saturated fatty acids obeyed saturation kinetics and a Lineweaver-Burk plot (Fig. 1B) confirmed this, giving an apparent K_m of $5\ \mu\text{M}$. Different batches of substrate gave K_m values ranging from 1 to $5\ \mu\text{M}$, probably due to differences in the original pantetheine concentration.

If end-product regulation exists, the presence of exogenous saturated fatty acids would affect the endogenous synthesis. The effects of pantetheine supplementation in medium with either $75\ \mu\text{M}$ oleic acid or $75\ \mu\text{M}$ oleic plus $75\ \mu\text{M}$ palmitic acid was therefore investigated (Table I). As before, growth increased markedly in the oleic acid cultures containing pantetheine. A small increase in cellular lipids was also evident in the palmitic/oleic acid plus pantetheine medium. In the oleic acid medium a low level of endogenous saturated fatty acid synthesis was apparent in the absence of pantetheine whereas pantetheine supplementation increased this synthesis dramatically. In the oleic plus palmitic acid medium there was a likewise low biosynthetic activity, but the effect of pantetheine addition in this case was a significantly smaller increase in syn-

TABLE I

INCORPORATION OF ENDOGENOUSLY SYNTHESIZED AND EXOGENOUSLY SUPPLIED FATTY ACIDS INTO THE MEMBRANE LIPIDS OF *A. LAIDLAWII*, INFLUENCE OF PANTETHEINE AND PALMITIC ACID

Oleic (18:1_c) and palmitic (16:0) acids were added each at a concentration of 75 μM, pantetheine (pan) at 12 μM. Cells were grown for 18 h. Exogenously added fatty acids were labelled with ¹⁴C and endogenous synthesis was monitored by the incorporation of [³H]acetate. Analysis was made by liquid scintillation counting and gas-liquid chromatography. SFA, saturated fatty acids.

Supplementation to the growth medium	nmol of SFA synthesized	nmol of exogenous fatty acids incorporated	% endogenous SFA in lipids	Total % SFA in lipids
18:1 _c	27.7	466.5	5.6	5.6
18:1 _c + pan	984.4	670.8	59.5	59.5
16:0 + 18:1 _c	99.7	1326.2	7.0	56.6
16:0 + 18:1 _c + pan	357.8	1440.2	22.6	62.6

thesis. These data suggest a possibility to control the activity of the fatty acid biosynthetic machinery at will, by the use of pantetheine and palmitic acid.

Effects of an increased fatty acid synthesis on the synthesis of membrane lipid species

Acetate is a normal catabolite in *A. laidlawii* [16]. Since the size of the endogenous acetate pool is difficult to estimate, it was only possible to use the incorporation of [³H]acetate as a qualitative measurement of endogenous saturated fatty acid synthesis. The fatty acids were incorporated into all polar lipids as well as into the neutral lipid fraction. In order to estimate membrane polar lipid composition we used the incorporation data for exogenously added fatty acids, since the exact amounts of both labelled and unlabelled acids were known.

Fig. 2. shows how the membrane molar ratio monoglucosyldiacylglycerol/diglucosyldiacylglycerol was altered in cultures grown for 18 h in media with

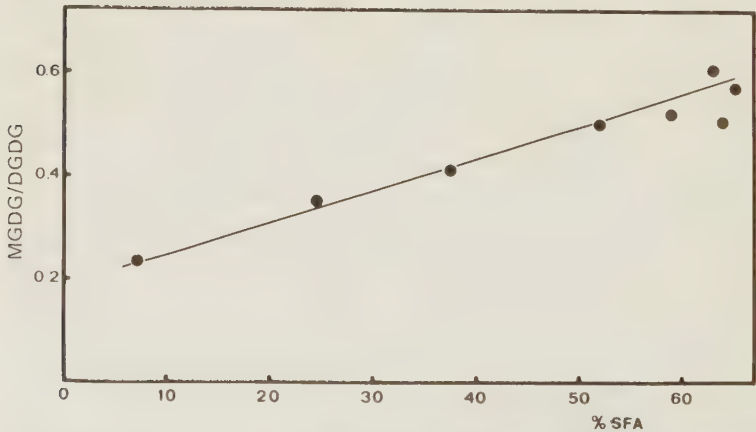


Fig. 2. Molar ratio between monoglucosyldiacylglycerol (MGDG) and diglucosyldiacylglycerol (DGDG) for membranes with different amounts of endogenously produced saturated fatty acids. The membranes were obtained by growing cells for 18 h in 50 μM oleic acid medium supplemented with different concentrations of pantetheine. Lipid amounts were estimated by liquid scintillation counting.

different pantetheine concentrations. These thus have different degrees of fatty acid saturation (cf. Fig. 1). Under the growth conditions employed, there was a linear relationship between the percentage of saturated fatty acids in the membrane lipids and the glucolipid ratio,

The ratios in Fig. 2 were compared with the ratios obtained in the presence of exogenous palmitic acid (Table II). An increase in the content of saturated fatty acids (Table I) yielded a higher monoglucosyldiacylglycerol/diglucosyldiacylglycerol ratio (Table II) regardless of the origin of the fatty acids. The values obtained are in good agreement with the results in Fig. 2. Thus there is no qualitative difference in the effect of fatty acids from different sources.

In addition to the dominating, electrically neutral (actually weakly anionic [5,17]) glucolipids, the membrane of *A. laidlawii* contains charged (strongly anionic) lipids, i.e. phosphatidylglycerol and glycerophosphoryl derivatives of mono- and diglucosyldiacylglycerol [3]. Table II reveals that when increasing the degree of fatty acid saturation in the lipids, there was a decrease in the total amounts of charged lipids. The same relationship was found after a successive increase in the saturated fatty acid content by supplementation of pantetheine to oleic acid medium (data not shown) (cf. Fig. 1).

When grown under normal conditions, *A. laidlawii* contains trace amounts of a glucolipid X (containing glycerol, glucose and fatty acids in the proportion 1 : 1 : 4) [2,3]. When grown in 50 μ M oleic acid medium with 12 μ M pante-theine, lipid X increased to approximately 10% (mol/mol) of the polar lipids. Typically this lipid always contained a very high degree of saturated fatty acids (approx. 90% mol/mol).

In the presence of pantetheine in the growth medium the cell membrane of *A. laidlawii* was brilliantly yellow in contrast to the greyish-white colour in its absence. The coloured compound was a neutral lipid and was identified as a carotenoid, probably neurosporene, on basis of its absorption spectrum in hexane (absorption maxima at 405, 432, 467 nm) (cf. Ref. 18). Examination of the other neutral lipids revealed an increase in the proportion of free fatty acids of endogenous origin. The total proportion of neutral lipids in the membrane did not increase substantially.

TABLE II

EFFECT OF PANTETHEINE AND PALMITIC ACID ON THE SYNTHESIS OF GLUCOLIPIDS AND CHARGED LIPIDS

The values refer to the same experiment as in Table I. See Table I for abbreviations. Molar ratio MGDG/DGDG is the molar ratio between monoglucosyldiacylglycerol (MGDG) and diglucosyldiacylglycerol (DGDG). The lipids used for the percent charged lipids of total data are: glycerophosphoryl monoglycosyldiacylglycerol, glycerophosphoryl diglucosyldiacylglycerol and phosphatidylglycerol.

Supplementation to the growth medium	Molar ratio MGDG/DGDG	% charged lipids of total (mol/mol)
18:1 _c	0.26	28.5
18:1 _c + pan	0.53	24.5
16:0 + 18:1 _c	0.62	23.1
16:0 + 18:1 _c + pan	0.68	21.9

The lipids used for the percent charged lipids of total data are: glycerophosphoryl monoglycosyldiacylglycerol and phosphatidylglycerol.

Induction of endogenous fatty acid synthesis by pantetheine during growth

The previous experiments all indicated that pantetheine supplementation to the lipid-depleted growth medium stimulated the synthesis of saturated fatty acids. The resultant increase in membrane molecular ordering [19] was accompanied by an increase in the monoglucosyldiacylglycerol/diglucosyldiacylglycerol ratio. The effects of temperature on this ratio has been investigated earlier in temperature-shift experiments [4]. Analogous experiments were now performed with pantetheine. A 50 μM oleic acid culture was grown at 37°C for 12 h. At this time the culture was divided in two parts. One part was supplemented with 12 μM pantetheine and the other remained unsupplemented. After supplementation there was an immediate increase in the rate of growth of the pantetheine culture (Fig. 3) and the cell membranes rapidly turned yellow. The saturated fatty acid content of the lipids also increased from 6 to 61% (mol/mol) in 12 h (data not shown), whereas there was a decrease in fatty acid saturation in the unsupplemented culture. Fig. 4 shows the changes in the monoglucosyldiacylglycerol/diglucosyldiacylglycerol ratio during growth. Obviously the same response in the ratio can be obtained by stimulation of endogenous saturated fatty acid synthesis in *A. laidlawii* as was provoked by a rapid temperature decrease.

Membrane protein composition

Sodium dodecyl sulphate polyacrylamide electrophoresis revealed small differences in staining intensity for a few bands in cultures grown with different fatty acid supplementations as shown earlier [8]. However, addition of pantetheine during growth did not result in detectable changes in membrane protein patterns.

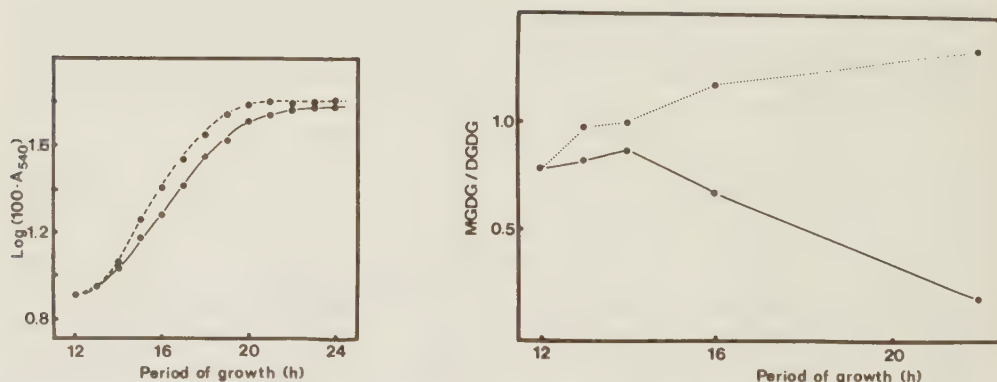


Fig. 3. Growth-promoting effect of pantetheine. A 50 μM oleic acid culture was divided in two parts after 12 h of growth at 37°C. One half was supplemented with 12 μM of pantetheine (hatched line) and the other remained unsupplemented (—). Growth was estimated by absorbance measurements at 540 nm.

Fig. 4. Changes in the molar ratio monoglucosyldiacylglycerol/diglucosyldiacylglycerol after stimulation of endogenous saturated fatty acid synthesis. After 12 h of growth at 37°C in medium containing 50 μM oleic acid, half of the culture was supplemented with 12 μM pantetheine (· · · · ·) while the other half remained unsaturated (—). Membrane polar lipid composition was analyzed at the times indicated (see Materials and Methods).

Discussion

A. laidlawii is capable of synthesizing saturated fatty acids from acetate in complex media [9]. From the results obtained with different *A. laidlawii* strains in our lipid-depleted medium, it is obvious that pantetheine can be a limiting factor for fatty acid synthesis, not only in partially defined media [14], but also in lipid-depleted complex media. By the use of extraction procedures which deplete the medium of pantetheine it is possible to virtually stop the endogenous saturated fatty acid synthesis. This procedure should make it possible to apply isotope techniques for accurate determination of fatty acid and lipid composition of many *A. laidlawii* strains. The method has been successfully used for *A. laidlawii* A (EF22) [3,4]. Inhibitors of fatty acid synthesis have been used to decrease endogenous synthesis and increase incorporation of exogenous fatty acids into *Acholeplasma* in studies of growth and fatty acid composition [20,21]. The use of a pantetheine-deficient medium may become a valuable complementary method in studies of lipid metabolism, since many inhibitors have toxic effects.

The mechanism of saturated fatty acid synthesis is well established for many organisms, e.g. *Escherichia coli* [22]. The malonyl-CoA pathway is probably at work in *A. laidlawii* too [11]. Acyl carrier protein plays an important role in this pathway, and a precursor, pantetheine, was found to stimulate acyl carrier protein activity of *A. laidlawii* substantially in a partially defined medium [14]. This probably occurred in our growth medium too, and a Lineweaver-Burk plot (Fig. 1b) revealed an apparent K_m of 5 μ M for the influence of pantetheine on fatty acid synthesis. For comparison, a pantothenate auxotrophic *E. coli* strain was found to require 2 μ M pantothenate to maintain exponential growth [23]. After addition of pantetheine during growth of *A. laidlawii* (Figs. 3 and 4) the onset of fatty acid synthesis was rapid, indicating that fatty acid synthetase may be a constitutive enzyme complex. Exogenous palmitate inhibited fatty acid synthesis in *A. laidlawii* A (EF22), confirming previous investigations on other strains [24,25]. An end-product regulation of fatty acid synthesis thus exists, although the efficiency of this mechanism has recently been challenged [25].

The addition of pantheteine resulted in a stimulation of carotenoid biosynthesis and the membranes turned yellow. Pantetheine is a precursor for coenzyme A, which in turn is needed in carotenoid biosynthesis. In media with pantetheine deficiency, the existing CoA is obviously spared for more important activities than carotenoid biosynthesis, which implies that carotenoids are not essential membrane components under all circumstances. Carotenoids are known to increase the molecular ordering in *A. laidlawii* membranes [26] and this has been suggested as a possible mechanism for a partial regulation of membrane fluidity [27].

Stimulation of the endogenous saturated fatty acid synthesis has a profound impact on polar lipid synthesis in *A. laidlawii*. Fig. 2 demonstrates the tightly coupled relationship which exists between the degree of fatty acid saturation and the monoglucosyldiacylglycerol/diglucosyldiacylglycerol ratio under the conditions described. These data give further evidence for the sensitivity of this lipid polar head regulatory mechanism. The regulation is, however, not sensitive

to fatty acid saturation per se, since a decrease in temperature during growth [4] gives qualitatively the same increase in the glucolipid ratio as the stimulation of saturated fatty acid synthesis caused by pantetheine (Fig. 4). Moreover, the glucolipid ratio is not coupled to any specific growth phase (Fig. 4 and Ref. 4). These experiments therefore indicate that the regulation mechanism senses the molecular ordering in the membrane. X-ray diffraction and $^2\text{H}_2\text{O}$ NMR measurements revealed that monoglucosyldiacylglycerol and diglucosyldiacylglycerol differ considerably in physical properties. Thus, pure monoglucosyldiacylglycerol exists as a non-bilayer phase (reverse hexagonal), whereas diglucosyldiacylglycerol has a lamellar phase structure [5]. Membrane lipid mixtures have lamellar phase appearances, this being the only phase structure compatible with the existence and function of biological membranes. It is possible that the regulation mechanism in *A. laidlawii* might be a similar method to regulate membrane stability of this mixture of components with different phase behaviours, as recently suggested for phosphatidylethanolamine from natural sources [28]. Phosphatidylethanolamine can change between different phase structures depending on temperature and fatty acid composition [28]. In light of its high saturated fatty acid content, the appearance of the apolar monoglucolipid X (which has a lamellar phase structure [5]) might be yet another regulating or scavenging mechanism (cf. [3]). Evidence for the existence of mechanisms sensitive to membrane molecular ordering have been obtained in *Tetrahymena pyriformis* for the fatty acid desaturase, which is regulated by membrane fluidity [29]. Furthermore, the importance of the lipid polar head groups for the regulation of membrane fluidity has been recently demonstrated for murine fibroblasts (LM cells) [30]. LM cells are incapable of synthesizing phosphatidylcholine from phosphatidylethanolamine. Supplementation of the culture with different choline analogues resulted in incorporation of these precursors and fatty acid compositions intermediate to ethanolamine and choline-supplemented cultures. A reverse mechanism sensitive to molecular ordering and affecting polar lipid head group composition is equally possible in *A. laidlawii*. Furthermore, differences in growth temperature affected the proportions of the individual phospholipids in *Pseudomonas halosaccharolytica* [31], giving further strength to the concept that different polar head groups may have important regulatory functions in membranes.

The degree of fatty acid saturation of *A. laidlawii* lipids also affected the proportion of charged lipids in the membrane (Table II). An increase in the amount of *cis*-unsaturated fatty acids (i.e. oleic acid) into the lipids makes the lipid molecules more bulky in the apolar part, probably leading to a decrease in membrane stability (cf. Ref. 32). Furthermore, unsaturated lipids will increase the lipid lateral packing area [33] and a decrease in the surface charge density will occur [34]. It is possible that the cell compensates for these changes by an increased synthesis of charged lipids, thereby restoring surface charge density and bilayer stability. Experimental evidence for this proposed mechanism in strain EF22 has indeed been observed after fatty acid manipulation [3] as well as temperature shiftdown [4]. In mutants of *Neurospora crassa* defective in lipid synthesis, it was found that albeit great differences in lipid composition in different mutants, the ratio of zwitterionic (weakly anionic) to the anionic

lipids was always constant [35], indicating the importance of constant membrane charge. Furthermore, the same balance seems to be of vital importance in *E. coli* [36]. The concept of constant surface charge density in *A. laidlawii* will be further investigated in future experiments. However, our results indicate that membrane polar lipid composition is regulated on at least two levels, i.e. the monoglucosyldiacylglycerol/diglucosyldiacylglycerol ratio and the balance between neutral and charged lipids.

In conclusion, the opportunity to control fatty acid synthesis *A. laidlawii* by the addition of pantetheine constitutes a powerful tool for investigations on the regulation and role of different lipid polar head groups in these membranes.

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Membrane Lipid Metabolism in *Acholeplasma laidlawii* A EF 22

Influence of Cholesterol and Temperature Shift-Down on Incorporation of Fatty Acids and Synthesis of Membrane Lipid Species

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1. Membrane lipid metabolism in *Acholeplasma laidlawii* A EF 22 has been studied under different conditions by applying three different techniques for changing membrane viscosity: fatty acid and cholesterol supplementation and temperature changes.

2. The molar relationship between the two dominating membrane lipids, monoglucosyldiglyceride and diglucosyldiglyceride, is to a large extent determined by membrane viscosity properties. This is shown by the varying metabolic responses occurring during incorporation of different fatty acids with and without cholesterol and by temperature shift-down experiments. Higher viscosity in membranes stimulates synthesis of monoglucosyldiglyceride at the expense of diglucosyldiglyceride. Synthesis of phospho and phosphoglucolipids is affected as well.

3. Temperature shift-down from 37 °C to 17 °C results in an immediate synthesis of monoglucosyldiglyceride accompanied by an increased incorporation of unsaturated fatty acids into this lipid. Synthesis of the other membrane lipid species (containing more unsaturated fatty acids) lags behind temporarily.

4. Incorporation from an equimolar mixture of palmitic and oleic acids together with cholesterol yields greater amounts of oleic acid in membrane lipids than incorporation in the absence of cholesterol, indicating that incorporation is viscosity dependent.

5. Studies of precursor relationships reveal that all main lipids have an active turnover which differs depending on membrane composition and conditions. Furthermore, this turnover proceeds with different intra-lipid pools.

6. Isolated membranes contain no detectable lipolytic enzymes capable of hydrolyzing membrane phospho or glycolipids. It is suggested that lipid turnover is partly mediated by enzymatic inter-lipid conversions, thus not allowing intermediates to accumulate.

The reason for the heterogeneity of lipids in biological membranes is not fully understood. One important function of different lipids is to maintain topological and transverse asymmetry within the membranes [1]. The regulation of different lipid enzyme systems is probably an important tool in structural differentiation of membranes.

The cytoplasmic membrane of *Acholeplasma laidlawii* has been the subject of many studies regarding the influence of fatty acid composition on membrane permeability, viscosity and phase transitions [2]. Despite these studies, relatively little is known about

the metabolism of the various lipid species, especially the glucolipids, which comprise a majority of the membrane lipids. Furthermore, the dynamic role of these glucolipids in the lipid bilayer is unknown.

We have earlier found both qualitative and quantitative variations in membrane lipid species in *A. laidlawii* strain EF 22 as a consequence of incorporation of different fatty acids into the membrane lipids [3]. The changes could be tentatively correlated with the physical properties of the fatty acids and their probable impact on membrane viscosity [3]. The packing properties of these lipids in the membrane bilayer are very similar [4]. Assuming a certain fatty acid composition, it is known from model systems that bilayer viscosity can also be modified by temper-

Enzymes. Glycerolphosphate acyltransferase (EC 2.3.1.15); Phospholipase A₁, A₂ (EC 3.1.1.4); Phospholipase D (EC 3.1.4.4).

ature changes and/or by incorporation of a modifying agent like cholesterol. Qualitatively, viscosity changes caused by the two latter modifications are probably not different from those caused by different fatty acids, although for cholesterol the liquid crystalline phase conditions of the bilayer is important [2]. In addition, a very important feature of cholesterol recently found in model system experiments is its ability to interact more specifically with certain lipids (or lipid fatty acid variants) than with others [5, 6]. This capacity is probably of great biological significance.

For *Escherichia coli*, temperature shift experiments have revealed a temperature-dependent acyl transferase activity for incorporation of fatty acids into membrane lipids [7]. Key steps in the multi-enzyme fatty acid synthesis system are probably also temperature-dependent as shown by the ratio of saturated and unsaturated fatty acids produced at different temperatures [8]. The result of these mechanisms is an adaption called 'homeoviscous' which maintains membrane viscosity of *E. coli* at a constant level regardless of growth temperature [9].

Most of the membrane fatty acid studies of *A. laidlawii* have only dealt with the overall incorporation of fatty acids into membrane lipids. From these studies it has become evident that the endogenous enzyme system for fatty acid synthesis has a very restricted capacity when compared to that of *E. coli*. Since *A. laidlawii* cannot synthesize unsaturated fatty acids, which are quite growth promoting, regulation of membrane viscosity probably must rely on selective incorporation of exogenous acids or on a differential synthesis of saturated acids with similar properties, i.e. short-chain fatty acids [2, 10].

In the present paper we have used the temperature shift-down technique as a tool to study the regulation of incorporation of fatty acids into the different membrane lipids as well as the synthesis of the entire lipids in the presence or absence of cholesterol. These conditions enable a study of the enzyme mechanisms involved due to their rapid activation by the large environmental change. Furthermore, the presence or absence of positional lipid fatty-acid variants, i.e. saturated/saturated, saturated/unsaturated and unsaturated/unsaturated lipid species, yields information about lipid metabolic pathways and precursor pools.

The results indicate that besides a temperature and viscosity dependent increase in degree of unsaturation which was different in different lipids, there were large and significant changes in relative amounts of the major glucolipids and phospholipids. All changes occurred much more rapidly in one of the glucolipids, monoglucosyldiglyceride. Part of these results have been presented at the joint Biochemical Society/Swedish Biochemical Society meeting in Aberdeen, March 1977.

MATERIALS AND METHODS

Growth Conditions

Acholeplasma laidlawii A, strain EF 22, obtained from Prof. E. A. Freundt (FAO/WHO Mycoplasma Reference Laboratory, Aarhus, Denmark) was grown statically in a tryptose/bovine serum albumin medium which contained 20 g lipid-depleted tryptose [11], 4.0 g lipid-depleted bovine serum albumin (Cohn V) [11], 7.0 g Tris, 0.5 g thallos acetate and 10000 I.U. penicillin G (Sigma Chemical Co.) per liter at pH 8.4. Each liter of medium was supplemented with either 75 μ mol of palmitic acid plus 75 μ mol oleic acid or with 150 μ mol oleic acid (Sigma) in ethanolic solution. These supplements were also given together with 8 mg/l of cholesterol (Sigma) which was added mixed with the fatty acids to avoid precipitation in the medium [11]. To label the membrane lipids 30 μ Ci [3 H]palmitic acid (57.9 Ci/mol) and/or 10 μ Ci [14 C]-oleic acid (59.7 Ci/mol) (the Radiochemical Centre) was added per liter.

Fresh medium was inoculated with 5% (v/v) of a 24-h 37 °C culture and grown in a water bath for 12 h at 37 °C in side-arm flasks. The culture was then divided in two portions; one of which was rapidly shifted to 17 °C in a water bath, while the other was maintained at 37 °C. Growth was followed by absorbance measurements at 540 nm. Samples for lipid and protein analysis were withdrawn at intervals and centrifuged at 32000 $\times g$ for 10 min at 20 °C in a Beckman J 21 centrifuge. The cells were washed once in β -buffer (150 mM NaCl, 50 mM Tris, 10 mM mercaptoethanol, adjusted to pH 7.4 with HCl) and freeze-dried.

Preparation of Membranes

Untreated cells washed once in β -buffer were osmotically lysed in deionized water by stirring for 45 min at room temperature. Membranes were collected by centrifugation at 32000 $\times g$ for 45 min at 5 °C and washed twice in β -buffer diluted 1/20 (v/v) in deionized water.

Preparation of Lipids

Freeze-dried cells or membranes (3–8 mg) were extracted once with 14 ml of chloroform/methanol 2/1 (v/v) for 1 h at room temperature under constant stirring. The lipid extract was taken to dryness under a stream of nitrogen and purified from non-lipid contaminants by passage through a Sephadex G25 Fine column in chloroform/methanol/water (60/30/4.5, v/v/v) [12].

Extracts were again evaporated to dryness under a stream of nitrogen with slight heating and dissolved in a small volume of chloroform/methanol (2/1, v/v). Individual lipid species were further separated by thin-

layer chromatography on 1.0 mm Silica gel H (Merck) plates containing 1% (w/v) $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$ in the solvent system chloroform/methanol/water (65/25/4, v/v/v) or on 0.5 mm Silica gel H plates in the solvent system light petroleum (b.p. 40–60 °C)/diethyl ether/acetic acid (glacial) (80/20/1, v/v/v).

Lipid spots were visualized by iodine vapour and by autoradiography on Kodirex X-ray film (Kodak). Identification procedures for the lipids have been described elsewhere [3]. Each lipid spot was scraped into a Pasteur pipette column stoppered with glass wool and eluted with chloroform/methanol (2/1, v/v) followed by methanol/formic acid (96/4, v/v) directly into a scintillation vial, and the solvents were evaporated under a stream of warm air.

Analytical Methods

Protein was determined by the method of Lowry et al. [13] with bovine serum albumin (Cohn V) as standard.

Fatty acid composition and total amount of each lipid were determined by liquid scintillation counting (Nuclear Chicago, Mark II/Searle) in an Omnifluor/toluene cocktail (New England Nuclear Company). Conversions of counts per minute to disintegrations per minute were obtained using known quenched ^3H and ^{14}C standard series (Radiochemical Centre).

Cholesterol was determined in the purified total lipid extract according to Rudel and Morris [14].

The temperature range of membrane lipid phase transition was determined by measurement of absorbance (500 nm) versus temperature [15,16] for liposomes in a Unicam spectrophotometer equipped with a temperature-controlled cuvette holder. The temperature was continuously changed and was measured in the cuvette with a thermistor connected to a precision temperature measurement unit (Knauer). Absorbance and temperature were plotted simultaneously on a recorder. Liposomes were prepared by sonification of 0.2–0.5 mg lipid per ml 0.150 M KCl in screw-capped tubes filled with N_2 . Sonification was carried out at constant temperature (40 °C) in a bath-type sonifier for 30 min. For comparison, liposomes of diglucosyldiglyceride from *A. laidlawii* containing biosynthetically incorporated [^2H]palmitic acid and physically characterized by nuclear magnetic resonance studies [17], were used as a transition temperature range standard.

Assay of Endogenous Lipolytic Activity

Washed membranes labelled with [^3H]palmitic and [^{14}C]oleic acid were suspended in β -buffer diluted 1/20 in H_2O (0.6 mg membrane protein/ml). Either no additions was made to the reaction mixture or one of the following was added: 5 mM Ca^{2+} or

0.11 mg Triton X-100/ml, or 5 mM Ca^{2+} + 0.11 mg Triton X-100/ml. Incubation was performed at 37 °C, and at intervals samples were withdrawn and centrifuged at $32000 \times g$ for 45 min at 5 °C. The membranes and supernatant were then freeze-dried. The dried specimen was extracted for lipids and the extract purified and analysed as described above.

RESULTS

Earlier results showed that large amounts of a saturated fatty acid in membrane lipids of *A. laidlawii* A EF 22 increased the relative amount of a membrane glucolipid, monoglucosyldiglyceride, at the expense of another glucolipid, diglucosyldiglyceride. A *cis* mono-unsaturated fatty acid reversed this relationship [3]. Despite the fact that the monoglucolipid is the biosynthetic precursor of the diglucolipid [18] and that these lipids are structurally similar, X-ray diffraction and nuclear magnetic resonance studies have revealed striking differences in their phase behavior [17]. This suggests different physiological functions of these lipids.

Absorbance and the Production of Cell Protein

Growth at 37 °C proceeded similarly with different fatty acid and cholesterol supplements when measured spectrophotometrically (Fig. 1). After temperature shift to 17 °C all cultures continued to grow albeit at a reduced rate. Following a lag of approximately 3 h, the palmitic plus oleic acid culture began to grow at the same rate as the other 17 °C cultures.

Despite the similarities in absorbance, cell protein contents of the 37 °C cultures were very different (Fig. 1). In particular the culture supplemented with palmitic plus oleic gave higher cell yields. After temperature shift all cultures except the one supplemented with palmitic plus oleic acids and cholesterol (Fig. 1B) showed a temporary halt or decrease in protein content (Fig. 1).

Lipid Composition before Temperature Shift

In *A. laidlawii* cellular lipids belong almost exclusively to the cytoplasmic membrane [2]. Under the growth conditions used here our strain has virtually no endogenous (saturated) fatty acid synthesis after 12 h of growth [3]. Exogenously supplied radioactively labelled fatty acids are proportionally incorporated into membrane lipids. These facts enabled us to determine amounts of membrane lipid species and their fatty acid content with high precision.

Table 1 shows the lipid composition for cells grown in differently supplemented media at the time of temperature shift-down (12 h). The main differences occurred in the amounts of monoglucosyldiglyceride

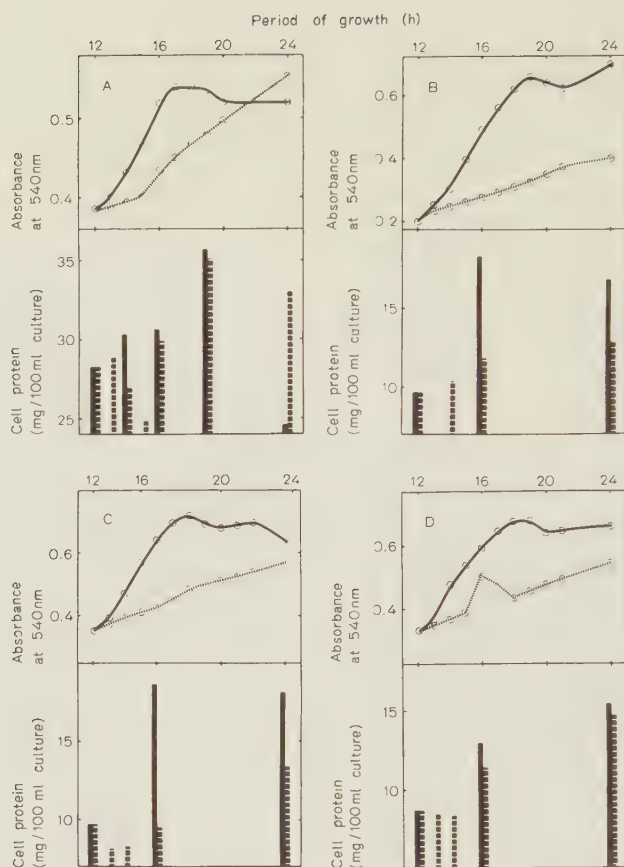


Fig. 1. Absorbance and protein content of *Acholeplasma laidlawii* cells after temperature shift-down. Cells were grown at 37 °C in medium with different fatty acid supplements. After 12 h one half of each culture was rapidly shifted to 17 °C while the other was maintained at 37 °C. Solid lines and bars; 37 °C cultures, hatched lines and bars; 17 °C cultures. The supplements were: (A) 75 μ M palmitic + 75 μ M oleic acid; (B) as in A plus 8 mg/l cholesterol; (C) 150 μ M oleic acid; (D) as in C plus 8 mg/l cholesterol.

and diglucosyldiglyceride as a response to fatty acid saturation and cholesterol incorporation. Large fluctuations in the syntheses of phospho and phosphogluco lipids were also evident. The main true phospholipid, phosphatidylglycerol, appeared in different amounts. Furthermore, the balance between gluco and phospho lipids was different in all experiments.

Lipid Changes in Cultures Supplemented with Palmitic plus Oleic Acids

In order to compare increases and/or decreases in separate membrane lipid species within the same or between different fatty acid and cholesterol supplements, each lipid species was assigned the relative value of 1.0 at 12 h (on basis of Table 1) and lipid

variations during subsequent growth were related to this figure.

Fig. 2A shows the changes occurring in the main membrane polar lipids; monoglucosyldiglyceride, diglucosyldiglyceride and phosphatidylglycerol, in growth medium containing palmitic and oleic acid. Immediately after the temperature shift, the amounts of diglucosyldiglyceride and phosphatidylglycerol decreased approximately 20% whereas the amount of monoglucosyldiglyceride remained constant. Absorbance and cell protein content (Fig. 1A) showed a 3-h lag. At the end of this lag the amount of monoglucosyldiglyceride started to increase substantially faster than the other two lipids. Between 8 and 12 h after the shift, lipid metabolism gradually yielded a lipid pattern similar to that at 37 °C except that the propor-

Table 1. *Lipid composition at the time of temperature decrease*
Cells were grown 12 h at 37 °C. Amounts of each lipid was quantified as described in Fig.2. Identification of the separate species was according to Materials and Methods

Lipid	Fatty acid supplement to growth medium			
	palmitic + oleic	palmitic + oleic + cholesterol	oleic	oleic + cholesterol
	mol/100 mol			
Glycerolphosphoryldiglucoacyldiglyceride	10.0	0.7	12.1	10.4
Glycerolphosphorylmonoglucoacyldiglyceride	4.0	2.1	3.6	3.4
Phosphatidylglycerol	19.1	20.3	25.7	34.7
Diglucoacyldiglyceride	21.3	31.0	29.3	31.2
Diphosphatidylglycerol	0.5	—	—	—
Monoglucoacyldiglyceride	39.8	42.7	27.9	18.6
Glucolipid X	4.7	1.8	1.4	1.7
Neutral fraction	0.7	1.3		

tion of glucolipids increased and phosphatidylglycerol and total phospholipids decreased (data not shown).

The addition of cholesterol (Fig. 2B) produced a somewhat different lipid pattern and changed the relationship between monoglucoacyldiglyceride and diglucoacyldiglyceride. The specific synthesis of lipids was approximately three times greater than without cholesterol. After temperature shift-down no metabolic lag or slowed increase in lipid amounts was observed for monoglucoacyldiglyceride and phosphatidylglycerol, whereas the relative amount of diglucoacyldiglyceride remained at the original level. This yielded an increased proportion of phosphatidylglycerol (and of total phospholipids, data not shown).

Lipid Changes in Cultures Supplemented with Oleic Acid

This supplement yielded a basic lipid pattern similar to that of the other acids at 37 °C (Fig. 2A, B). Addition of oleic acid only, resulted in a different lipid metabolism after the shift to 17 °C, however. The relative amount of diglucoacyldiglyceride increased only slightly (about 20%) whereas monoglucoacyldiglyceride increased two and a half times (Fig. 2C). Immediately after the temperature shift there was a rapid and pronounced (80% of the phosphatidylglycerol pool) decrease in phosphatidylglycerol. This temporary decrease was stoichiometrically correlated with a likewise temporary increase in diphosphatidylglycerol from 0–11 mol/100 mol (the latter is not shown in Fig. 2C).

The addition of cholesterol (Fig. 2D) caused a very pronounced lipid synthesis immediately after temperature decrease, but not synthesis of diphosphatidylglycerol. These large changes were also evi-

dent from the absorbance curves (Fig. 1D). The final lipid pattern was similar to that obtained without cholesterol.

Incorporation of Unsaturated Compared with Saturated Fatty Acids

In addition to changes in lipid composition, incorporation into the individual lipids of an unsaturated versus a saturated fatty acid was monitored to get a more fully detailed picture of temperature adaptation. Table 2 shows that during growth at 37 °C all main polar lipids contained different amounts of unsaturated fatty acid (oleic acid) and had different rates of incorporation. Final levels (24 h) were also dissimilar. The same features were found after addition of cholesterol (Table 2B), but the unsaturated fatty acid content was consistently higher.

After temperature decrease, incorporation was halted for 4 h at original levels for all lipids except monoglucoacyldiglyceride (Table 2A, B). The latter lipid showed an even more pronounced incorporation of oleic acid than at 37 °C. It is noteworthy that diglucoacyldiglyceride did not show an increased unsaturation ratio and that in phosphatidylglycerol there was no change until later stages of growth where unsaturation increased most profoundly. For all lipids except diglucoacyldiglyceride (Table 2A), final levels of oleic acid were always substantially higher at 17 °C than at 37 °C.

Membrane Lipid Phase Transition

According to McDonald et al. [15] pronounced variations in absorbance of liposome suspensions with small temperature changes correspond to a lipid phase transition. Fig. 3 indicates that the upper limit of the

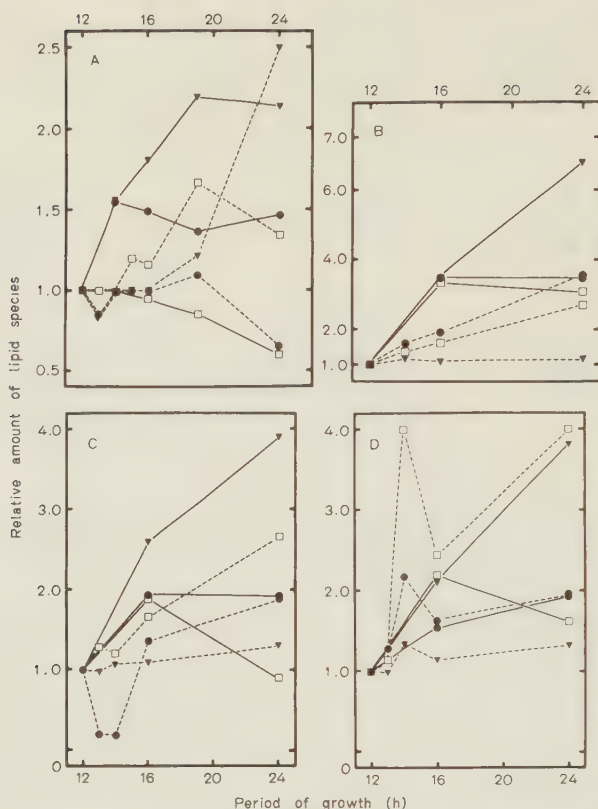


Fig. 2. Metabolism of major membrane polar lipids. Growth and temperature decrease procedures were as described in Fig. 1. The amount of each lipid at 12 h was assigned the relative value of 1.0, and lipid variations during subsequent growth was related to this figure. (See Table 1 for actual molar composition at 12 h.) Amount of each lipid was quantified in a liquid scintillation counter. (□) Monoglucosyldiglyceride; (▼) diglucosyldiglyceride; (●) phosphatidylglycerol. Solid lines, 37 °C; hatched lines, 17 °C. Supplements were (A) 75 μ M palmitic plus 75 μ M oleic acid; (B) as in A plus 8 mg/l cholesterol; (C) 150 μ M oleic acid; (D) as in C plus 8 mg/l cholesterol

gel to liquid crystalline phase transition for membrane lipid from cells grown in palmitic plus oleic acid extends from 25–34 °C for both 37 °C and 17 °C cells. Turbidity changes in the vicinity of 0 °C were most probably caused by changes in water structure connected with freezing. Diunsaturated lipid species are known to have their gel to liquid phase transition well below 0 °C. It has been shown earlier that deuterium-labelled diglucosyldiglyceride from this strain has the gel to liquid phase crystalline phase transition above 25 °C [17]. A good correspondence between nuclear magnetic resonance measurements and these optical measurements is evident. Growth at 37 °C thus occurred well above the gel to liquid crystalline phase transition temperature, whereas after temperature shift growth occurred in the middle of this transition.

Cholesterol Amounts in Membranes

In biological membranes cholesterol is considered to be part of the lipid matrix. The pronounced variations in membrane lipid composition in our strain might affect the uptake and distribution of cholesterol. Fig. 4 shows the ratio of cholesterol to total lipids (in arbitrary units) for cells grown with oleic or palmitic plus oleic acid at 37 °C and after shift to 17 °C. Obviously both the kind of fatty acid incorporated and temperature affected cholesterol uptake.

Lipid-Degrading Enzyme Activities in Isolated Membranes

The pronounced and sometimes rapid variations in amounts of different lipids and their individual

Table 2. Effect of temperature decrease on incorporation of unsaturated versus saturated fatty acids into the different major membrane lipid species
Temperature decrease after 12 h growth at 37 °C was accomplished as described in Fig. 1. During the growth conditions depicted, the sum of palmitic plus oleic acid was always approximately 100%. The figures below describe the fraction (mol/100 mol) of oleic acid in each lipid. The fatty acids were quantified as described in Fig. 2. Growth medium fatty acid supplements: (A) 75 µM palmitic + 75 µM oleic acid, (B) as in A plus 8 mg/l of cholesterol. n.d., not determined

A

Lipid	Incubation temperature	Oleic acid incorporated after growth time of			
		12 h	14 h	16 h	24 h
	°C	mol/100 mol			
Phosphatidylglycerol	37	37	55	57	61
	17	37	39	40	69
Diglucosyldiglyceride	37	41	45	48	47
	17	41	43	43	43
Monoglucosyldiglyceride	37	34	44	45	42
	17	34	40	45	50

B

Lipid	Incubation temperature	Oleic acid incorporated after growth time of			
		12 h	14 h	16 h	24 h
	°C	mol/100 mol			
Phosphatidylglycerol	37	56	n.d.	55	57
	17	56	56	55	74
Diglucosyldiglyceride	37	54	n.d.	56	58
	17	54	55	57	66
Monoglucosyldiglyceride	37	50	n.d.	52	47
	17	50	57	63	69

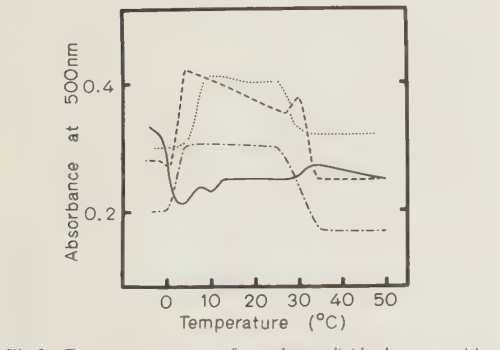


Fig. 3. Temperature range of membrane lipid phase transition. Total lipid extracts from palmitic plus oleic acid grown cells were sonicated in 0.15 M KCl at 40 °C in an atmosphere of N₂. Absorbance at 500 nm and temperature increase were measured simultaneously. Symbols: (—) lipid extract from cells grown 12 h at 37 °C; (----) lipid extract from cells grown 24 h at 37 °C; (.....) lipid extract from cells grown 12 h after decrease to 17 °C; (- - -) diglucosyldiglyceride (containing oleic acid plus ω -deuterium labelled palmitic acid) extracted from cells grown 24 h at 37 °C

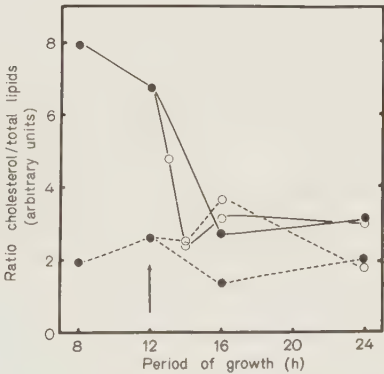


Fig. 4. Ratio of cholesterol to total lipids. Cholesterol was determined colorimetrically. Total lipid is expressed as the sum of disintegrations per minute for the individual lipids. Solid line: medium supplemented with 75 µM palmitic plus 75 µM oleic acid and 8 mg/l of cholesterol. Hatched lines: medium supplemented with 150 µM oleic acid and 8 mg/l of cholesterol. (●) 37 °C; (○) 17 °C. The arrow denotes the time of temperature decrease

Table 3. Incubation of membranes

Cells were grown for 12 h in medium supplemented with 75 μ M palmitic plus 75 μ M oleic acid. Membranes were prepared and incubated at 37 °C in dilute β -buffer (1/20) at a concentration of 0.6 mg membrane protein/ml. Samples for analysis were withdrawn at intervals between 0 and 19 h and centrifuged. The pellet and supernatant were freeze-dried and analyzed for lipid composition (see Materials and Methods)

Lipid	Membrane lipid composition after				
	0 h	5 h			
		no addition	Triton X-100	Ca ²⁺ Triton	Ca ²⁺
	°				
Glycerolphosphoryldiglucoacyldiglyceride	8.8	10.3	7.1	5.0	8.1
Glycerolphosphorylmonoglucoacyldiglyceride	2.5	3.4	2.6	1.3	3.2
Phosphatidylglycerol	17.9	13.9	18.2	14.1	12.5
Diglucoacyldiglyceride	21.8	21.1	23.7	20.8	20.6
Monoglucoacyldiglyceride	44.1	45.6	44.1	51.9	48.3
Glucolipid X	3.9	3.4	3.0	5.1	3.7
Neutral fraction	0.9	2.4	1.4	1.8	3.6
Fraction of total membrane and supernatant lipid	100.0	99.2	38	78	99.2

fatty acid composition in relation to the relatively slow growth rate of this organism suggested the presence of lipid-modifying enzymes. Existence of such enzymes is usually shown by pulse-chase techniques with radiochemicals. Since *Acholeplasma* cells are very fragile and not osmotically stable in buffer solutions, incubation in order to obtain the products of these enzymes had to be carried out with pure membranes. Small amounts of divalent cations or nonionic detergents are sometimes known to stimulate lipolytic enzymes [19]. Ca²⁺ and Triton X-100 were thus included in the assay. The concentration of Triton X-100 (0.11 mg/ml) was well below the critical micellar concentration [20] for this compound and corresponded to approximately 0.15 mol of Triton X-100 per mol of membrane lipid.

Table 3 shows that this organism probably did not contain any constitutive lipolytic enzymes (phospho or gluco lipases A1 or A2) like those found in *Mycoplasma hominis* [21] or *E. coli* [22] since no free fatty acids or lysolipids could be detected and no changes in saturation ratio occurred. Alternatively, the enzymes were not induced or unmasked by this assay procedure. The neutral fraction did reveal a small increase of liberated diglycerides. Phosphatidylglycerol exhibited losses probably caused by a phospholipase C or by metabolic conversion to other lipids.

In the combined Ca²⁺ + Triton X-100 incubation assay only a small fraction of monoglucoacyldiglyceride was released into the supernatant although this lipid occurred in largest amounts in the membrane. Larger amounts were released from the other lipids (data not shown). Neither Ca²⁺ nor Triton X-100 alone produced this effect. The stabilising effect of Ca²⁺ together with Triton X-100 is also seen when

compared with Triton alone (data not shown). In all assays lipids released into supernatants were slightly but significantly more unsaturated than the remaining membrane lipids.

DISCUSSION

It can be concluded from Table 1 that different fatty acid and cholesterol additions affect the syntheses of the various lipid species and thus give rise to differences in lipid composition of *A. laidlawii* cells. This implies that the physical properties of the membrane lipid bilayer are different in each case and that the effects of a temperature decrease will vary. The differences in lipid composition will affect the existence of 'bound' and 'free' lipid domains. The latter will cause structural dispositions for membrane protein micro-environments, including enzymes, as recently suggested by Wallace et al. [23].

The differences in physical environment will cause different metabolic responses for the membrane lipid syntheses in the four differentially supplemented cultures (Fig. 2). However, all have certain characteristic features. The most prominent general changes are obtained from the relative syntheses of monoglucoacyldiglyceride and diglucoacyldiglyceride. Monoglucoacyldiglyceride is considered to be the metabolic precursor of diglucoacyldiglyceride [18]. The immediate effect of the rapid temperature decrease for the different cultures is a rapid increase of the viscosity of the membrane lipid bilayer (Fig. 3). In addition, synthesis of monoglucoacyldiglyceride increased compared with diglucoacyldiglyceride after temperature change (Fig. 5A, B). This is in agreement with the viscosity-dependent regulatory mechanism for the relative syntheses of monoglucoacyldiglyceride and diglucoacyl-

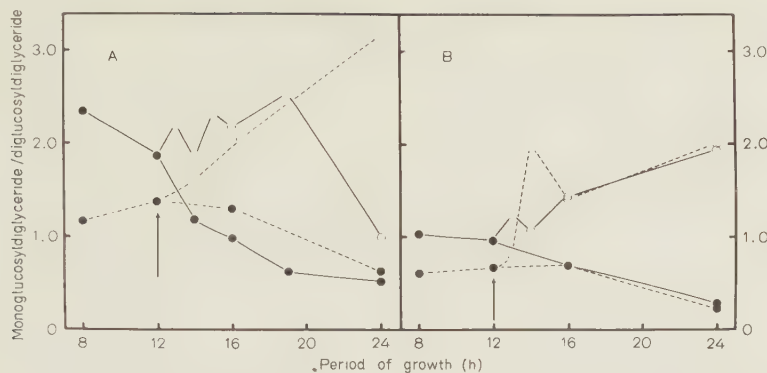


Fig. 5. Ratio of monoglucosyldiglyceride to diglucosyldiglyceride for different growth conditions. Growth and temperature decrease procedures were as described in Fig. 1. Quantification of the lipids was done as in Fig. 2. The fatty acid supplements were: (A) 75 μ M palmitic plus 75 μ M oleic acid; (B) 150 μ M oleic acid. Solid lines denote supplements with fatty acids, hatched lines supplements with fatty acids plus 8 mg/l of cholesterol. (●) 37 °C; (○) 17 °C. The arrows denote the time of temperature decrease

diglyceride proposed by us in earlier studies regarding the influence of different saturated and unsaturated fatty acids on these syntheses [3]. These findings are further strengthened by the effect of cholesterol reported here. In 37 °C cultures a higher degree of fatty acid saturation gives a higher monoglucosyldiglyceride/diglucosyldiglyceride ratio (Fig. 5A, cf. Table 2A). This ratio is lowered in the presence of cholesterol (Fig. 5A) during early growth phases. In cultures with palmitic and oleic acid the lipids characteristically have a high content of saturated fatty acids during these phases (particularly palmitic acid) [3]. The action of cholesterol here is probably to lower the viscosity of the dominating di-saturated lipid species which in turn affects the monoglucosyldiglyceride/diglucosyldiglyceride relationship. Continued growth yields increasing amounts of unsaturated fatty acid (oleic) in the lipids. Now cholesterol causes a higher monoglucosyldiglyceride/diglucosyldiglyceride ratio compared with the palmitic plus oleic acid supplement alone. Although with the former supplement (i.e. + cholesterol), the amount of oleic acid is higher in the lipids (Table 2A, B). An interaction of cholesterol with the dominating palmitic oleic and oleic/oleic acid lipid species is probably the cause. Consequently, the low endogenous production of saturated fatty acids during early growth with oleic acid supplements [3] yields mixed fatty acid lipid species, the viscosity of which is lowered by cholesterol. This yields a somewhat lower monoglucosyldiglyceride/diglucosyldiglyceride ratio than without cholesterol during early growth. In the logarithmic growth phase, interaction of cholesterol with the almost 100% diunsaturated lipid species is probably not enough to affect monoglucosyldiglyceride/diglucosyldiglyceride synthesis since maximal amounts of cho-

lesterol do not exceed 18 mol/100 mol. Furthermore, the growth temperature (i.e. 37 °C) is far above the critical lipid bilayer transition temperature [2].

The rapid temperature decrease from 37 °C to 17 °C causes an inversion of the relative rates of synthesis for monoglucosyldiglyceride and diglucosyldiglyceride (Fig. 2). This results in a rapidly increasing monoglucosyldiglyceride/diglucosyldiglyceride ratio (Fig. 5) both with and without cholesterol. Generally, monoglucosyldiglyceride is the first lipid which is synthesized in significantly larger amounts than pre-shift levels (Fig. 2). The non-regularity of the monoglucosyl/diglucosylglyceride ratio curves (Fig. 5) in periods following temperature shift indicates 'overshoot' phenomena in the regulation of these lipid balances. Such over-shoot phenomena have been observed in regulation of membrane lipid fatty acid composition in *E. coli* [24].

The delicate lipid balances are not restricted to monoglucosyldiglyceride or diglucosyldiglyceride. Both phosphatidylglycerol and phosphogluclipids are synthesized in significantly larger amounts in the oleic acid supplemented media (Table 1 and Fig. 2). Furthermore, the rate of phosphatidylglycerol synthesis is also temperature dependent (Fig. 2). With one of the supplements, oleic acid, temperature decrease rapidly activates a diphosphatidylglycerol synthetase, which depletes 80% of the phosphatidylglycerol pool and increases the diphosphatidylglycerol amounts from 0.5–11 mol/100 mol of total lipids (Fig. 2C) in 1 h. The diphosphatidylglycerol pool remains constant for another hour and is then rapidly reconverted to phosphatidylglycerol (Fig. 2C) presumably by a phospholipase D type of enzyme. Presence (and latency) of these enzymes can also be demonstrated by fusion of viable *A. laidlawii* cells

with small amounts of selectively labelled phosphatidylglycerol lipid vesicles (unpublished observation).

Cholesterol and temperature also affect incorporation profiles of saturated (palmitic) and unsaturated (oleic) acids (Table 2A, B). Growth at 37 °C yields different incorporation and different rates of change in all membrane lipids with time. The physiological background to this variance can be due to the following: different turnover rates, both between different lipids and within intramolecular variants of the same lipid [25,26]; selected enzymatic modification of existing lipids, i.e. deacylation (by lipases) followed by reacylation; the existence of different metabolic precursor pools for synthesis of different lipids [27]. None of these alternatives will exclude the others, but one of them may dominate. Temperature shift-down to 17 °C reveals that an immediate increase in oleic acid content is localized to monoglucosyldiglyceride, both with and without cholesterol (Table 2A, B). Table 2A and Fig. 2A further show that with monoglucosyldiglyceride and diglucosyldiglyceride amounts remaining at constant levels for 2 h after temperature shift in the palmitic plus oleic acid supplement, the unsaturated fatty acid content of monoglucosyldiglyceride is increasing from 34–40 mol/100 mol, probably by increased turnover or enzymatic modifications. Whereas diglucosyldiglyceride does not show increased unsaturated fatty acid content after temperature shift, phosphatidylglycerol does so markedly between 4 and 12 h afterwards. Here, the decrease in phosphatidylglycerol amounts at the same time indicates a rapid degradation (turnover) or modification. The same, but to a lesser extent, also holds for monoglucosyldiglyceride during later stages of growth at 17 °C. It is obvious from these results that *A. laidlawii* is equipped with a very subtle mechanism for regulating the degree of membrane lipid unsaturation as a response to temperature. Presence of cholesterol in the membranes yields a similar response in membrane lipid unsaturated fatty acid content, both with time and order of incorporation in the lipids. The overall incorporation is higher, however, both at 37 °C and at 17 °C. The unsaturated fatty acid content is most readily explained by cholesterol causing a viscosity increase when interacting with the dominating saturated/unsaturated lipid species. This viscosity increase is thus compensated by an increase in unsaturated fatty acid incorporation into the membrane lipids in analogy with the temperature-caused viscosity changes just mentioned.

Our studies indicate that *A. laidlawii* A, strain EF 22, is equipped with an adaptable and complex enzyme system for regulating lipid properties and balances in its membrane. We have explored here the impact of three different factors that affect membrane viscosity, i.e. fatty acid composition, temperature and cholesterol. All of them reveal both general and spe-

cial regulatory properties on membrane lipid metabolism. Furthermore, they show how potent and dynamic these effects may be (see for example the rapid lipid synthesis illustrated in Fig. 2D). Earlier studies regarding membrane lipid stability and turnover in *A. laidlawii* B have yielded contradictory results [28–30]. Since the results reported here strongly indicate both rapid turnover and modification we have therefore attempted to characterize the enzymes responsible for endogenous breakdown of the membrane lipids as earlier described for *E. coli* [22]. A membrane-bound lysophospholipase has also been found earlier in *A. laidlawii* strain B [31]. Our results show that either no constitutive degrading enzymes were present or that they were not activated or unmasked. Still it is obvious that turnover exists, probably by inter-lipid conversions by metabolic enzymes not allowing intermediates to accumulate. Differences in saturation ratios for different lipids (Table 2A, B) also strongly indicate that these conversions proceed with different precursor pools. It is known that monoglucosyldiglyceride is a precursor for the synthesis of diglucosyldiglyceride [18]. Table 2A and B show large differences in fatty acid composition between these lipids. Thus the glucosylation of monoglucosyldiglyceride to yield diglucosyldiglyceride proceeds with monoglucosyldiglyceride molecules containing different ratios of palmitic and oleic acid than the bulk monoglucosyldiglyceride lipids. Evidence for this proposed mechanism has also been obtained by fusion of *A. laidlawii* cells with different selectively double-labelled liposomes made from *A. laidlawii* lipids (unpublished observations).

Recent results from model systems indicate that cholesterol interacts preferably with some lipids compared to others [5]. These findings might explain the amounts of cholesterol found in different biological membranes as a function of the dissimilar lipid compositions of these membranes [6]. The differences in cholesterol content (Fig. 4) between differentially supplemented *A. laidlawii* cell membranes is thus probably a reflection of different lipid organisation. Results shown in Fig. 2 strongly favour such an interpretation. However, plots of cholesterol content versus amounts of the different lipids (data not shown) did not indicate linear relationships as earlier proposed by Razin [32]. Cholesterol uptake is probably dependent on the structural organisation of the lipids (i.e. lipid domains). Furthermore, the physical properties of membrane lipid fatty acids will affect this uptake [33–35]. The latter also holds for fatty acid uptake [36].

The very pronounced impact of the three factors affecting viscosity (fatty acids, temperature, and cholesterol) on membrane lipid biosynthesis, indicates that other cellular syntheses can be affected as well. Fig. 6 shows the relationship between lipids and cel-

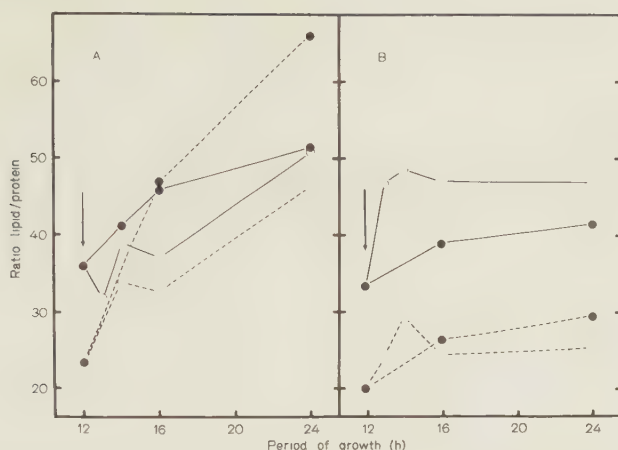


Fig. 6. Changes in the ratio of total lipid to cell protein. Protein was determined by the Lowry method. Total lipid is expressed as the sum of dis./min for the individual lipids. The ratios are given in arbitrary units. The fatty acid supplements were: (A) 75 μ M palmitic plus 75 μ M oleic acid; (B) 150 μ M oleic acid. Solid lines denote supplementations with fatty acids, hatched lines supplementations with fatty acids plus 8 mg/l of cholesterol. (●) 37 °C; (○) 17 °C. The arrows denote the time of temperature decrease

lular protein in different experiments reported here. Lipid amounts are also representative of amounts of membrane [3]. Generally, cholesterol decreases this ratio, i.e. decreases relative amounts of membranes. A rapid temperature decrease affects this relationship in opposite ways, probably depending on different membrane lipid properties. The over-all large variations of protein synthesis to changing membrane properties might be dependent on the fidelity of protein synthesis on the different membrane lipid matrixes [1,37] and on allosteric modifications of important enzymes.

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FRACTIONATION OF MEMBRANES FROM *ACHOLEPLASMA LAIDLAWII* A ON THE BASIS OF THEIR SURFACE PROPERTIES BY PARTITION IN TWO-POLYMER AQUEOUS PHASE SYSTEMS

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Summary

Acholeplasma laidlawii A consists of pleomorphic cell clusters surrounded by a single membrane. When lysed, a cell gives rise to several membrane fragments which cannot be separated from each other by isopycnic sucrose gradient centrifugation. A heterogeneous lateral organization of the cell membranes was detected by countercurrent distribution of membrane fragments in a two-polymer aqueous phase system. It revealed that the membranes consist of at least two subpopulations with respect to surface properties. Changes in the fatty acid and cholesterol content of the membranes revealed that the resolution of different subpopulations was predominantly due to a critical ratio of monoglucosyldiglyceride to diglucosyldiglyceride. The heterogeneity of the membrane probably depends on lipid-lipid and lipid-protein steric interactions. Charged lipids, an apolar monoglucolipid and the ratio between lipids and proteins also affect membrane partition. The differences in the subpopulations were further reflected by different specific activities of NADH dehydrogenase, NADH oxidase and ATPase. These activities varied independently. Minor quantitative differences in the protein patterns of different subpopulations were apparent. The origin and the preservation of the membrane subpopulations are discussed in terms of lipid-lipid and lipid-protein interactions, their age and energy metabolism.

Introduction

Although biological membranes have been much studied, relatively little information is available on membrane organization in the lateral plane. Because of their physical properties, lipids are known to affect the localization and

sometimes the activity of the membrane proteins. The gel to liquid crystalline phase transition in *Acholeplasma laidlawii* membranes causes an aggregation of integral proteins as visualized by freeze-etch electron microscopy [1,2]. Surface proteins are affected as well [3]; and the interactions between a certain lipid (phosphatidylglycerol) and membrane proteins are changed (summarized in ref. 4).

In larger bacteria, e.g. *Escherichia coli*, a similar behaviour is observed with respect to protein aggregation [5]. Preparation of membranes from *E. coli* yields small vesicles of inner and outer membranes. Low temperature during these steps produce vesicles with large differences in protein-lipid ratio due to lateral phase separation [6,7]. Such vesicles can be separated by isopycnic centrifugation. The two or three membrane fractions obtained differ, in addition to lipid-protein ratio, with respect to protein composition, and ratio of saturated to unsaturated fatty acids. There is no change in lipid species [7].

In *A. laidlawii* we have found a regulatory system that varies the relative amounts of membrane polar lipid species in response to induced variations in membrane viscosity [8,9]. These metabolic changes proceed with different intralipid species and turnover rates [9]. The physical properties of the involved (glyco)lipids differ significantly [10]. Furthermore, loosely-bound molecules at the membrane surface in *A. laidlawii* affect the lateral packing properties of the lipids [11,12]. All this indicates the probability of a heterogeneous lateral membrane organization in this organism.

The co-ordination between DNA-replication and cell division in *A. laidlawii* is loose, causing the cells to normally grow in clusters surrounded by the same, single cell membrane [13]. If lateral heterogeneities exist, there will be differences in composition for different parts of a membrane enclosing these cell entities. Such an organization will cause different membrane regions to have different surface properties. Two-polymer, aqueous phase systems have proved to be very useful for the separation by partition of cells and membrane vesicles differing only slightly in surface properties [14–16]. Recently, partition studies have been successfully extended to include liposomes [17]. We report here on the existence of different membrane fractions from *A. laidlawii*, separated by countercurrent distribution in two-polymer aqueous phase systems.

Materials and Methods

Organism and growth conditions

A. laidlawii A, strain EF22 [8], was grown for 18 h at 37°C in a lipid-depleted bovine serum albumin-tryptose medium [8]. The medium was supplemented with palmitic and oleic acids in different amounts, the sum of which was always 0.150 mM. The ratios of palmitic to oleic acid used were 120/30, 90/60, 75/75, 30/120 and 0/150. The latter supplement was also given together with 0.020 mM cholesterol. Membrane lipids were radioactively labelled by adding 30 $\mu\text{Ci/l}$ [^3H]palmitic acid, and/or 10 $\mu\text{Ci/l}$ [^{14}C]oleic acid and, when needed, 40 $\mu\text{Ci/l}$ [^3H]cholesterol, (Radiochemical Centre, Amersham, England).

Cells were harvested by centrifugation at 11 000 $\times g$ for 20 min at 5°C and

washed once in cold β -buffer. Membranes were prepared by osmotic lysis at 22°C for 45 min during agitation and collected by centrifugation at 32 000 $\times g$ for 45 min at 5°C. They were washed twice in 0.010 M sodium phosphate buffer, pH 7.4, and kept at 2°C for not more than 1 h before continuing with the procedure (see below). Quantities of membranes were estimated by absorbance measurements at 500 nm in 15 ml 0.010 M sodium phosphate buffer and expressed as absorbance units, (i.e. one unit is equal to an absorbance of 1.0 for 15 ml membrane suspension read in a 1 cm cuvette).

Countercurrent distribution

In preliminary experiments an appropriate phase system for *A. laidlawii* was selected as described by Walter [18].

The phase system was composed of 5% (w/w) Dextran T500, M_r 500 000 (Pharmacia, Batch no. 5556), 4% (w/w) poly(ethylene-glycol), M_r 6000, trade name Carbowax 6000 (Union Carbide), 8.3 mmol sodium phosphate buffer, pH 7.4, and 3 mmol NaCl, per kg final mixture. After mixing the components, the phases were allowed to equilibrate overnight at 3°C. The top and bottom phases were then separated and stored at 3°C.

Washed membrane preparations corresponding to 0.50 absorbance units were suspended in 4.50 ml of top phase (load mix). An automatic thin-layer countercurrent distribution apparatus with 120 cavities was used [19]. Cavities 0–3, 40–43 and 80–83 each received 0.5 ml of bottom phase and 0.9 ml of one of the load mixes. All other cavities received 0.6 ml bottom phase and 0.8 ml top phase. 39 transfers were completed at 3°C using a settling time of 6.5 min and a shaking time of 25 s.

Analysis of membranes

After the countercurrent run, membranes from each cavity were collected directly into plastic tubes or, when chemical and enzymatic analyses were to follow, 1.6 ml 37 mM 2-mercaptoethanol was first added to each cavity to convert the phase system to a homogenous (one-phase) suspending medium.

Absorbance of membranes was measured at 500 nm. Protein content was estimated [20] after precipitation of the membranes with cold trichloroacetic acid according to Albertsson [21]. Radioactivity of the labelled membranes was determined by liquid scintillation counting [9] after digestion of the membranes in Protosol (New England Nuclear Co.) overnight. cpm were converted to dpm using known quenched ^3H and ^{14}C standards.

On the basis of the distribution patterns measured by absorbance, some adjacent tubes were pooled separately (see Figs. 1 and 4). These pooled membranes were freed from adhering polymers by three consecutive washes in β -buffer diluted 1 : 20 with deionized water. Pellets were dispersed in a small volume of the same buffer and stored at -70°C . Samples for enzyme assays were also made 20 mM with respect to 2-mercaptoethanol.

Lipids were extracted with the Bligh and Dyer method according to Kates [22]. Separation of lipids was performed by thin-layer chromatography (Silica gel H, Merck) on borate-buffered plates [8] in chloroform/methanol/water (65 : 25 : 4, v/v). Radioactivity was measured by liquid scintillation counting [9].

Protein patterns of different membrane fractions were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis according to Neville [23] as modified by Jergil and Ohlsson [24]. The gels were cast in $9.5 \times 7.5 \times 0.3$ cm slab gel forms. Upper stacking gel contained 3.5% (w/w) acrylamide (6.5% crosslink) and the separation gel 11.8% (w/w) acrylamide (1% cross-link). Membrane samples were solubilized at 100°C for 3 min in double strength upper reservoir buffer [23] containing 3.8% (w/v) sodium dodecyl sulphate. Electrophoresis was carried out for 4 h at a constant current of 20 mA per gel. After the run, the gels were stained overnight with Coomassie Brilliant Blue R250. The following reference proteins were used for molecular weight determinations: lysozyme, DNAaseI, ovalbumin and bovine serum albumin.

Enzyme assays

NADH dehydrogenase (EC 1.6.99.3) activity. Membranes were treated with 2.5% (w/v) sodium deoxycholate in 6.36 mM tris(hydroxymethyl)amino-methane-HCl buffer, pH 7.4, containing 1.27 mM 2-mercaptoethanol for 45 min at room temperature. $\text{K}_3[\text{Fe}(\text{CN})_6]$ was used as artificial electron acceptor. Final concentrations were: 50–250 μg membrane protein, 1.00% (w/v) sodium deoxycholate, 2.50 mM Tris-HCl (pH 7.4), 0.50 mM 2-mercaptoethanol, 7.80 mM NaCl, 1.80 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ and 0.120 mM NADH, in a volume of 1.20 ml. The reaction was measured continuously at 420 nm in a spectrophotometer at 37°C . Controls of any spontaneous reaction were also run. Enzyme activity was calculated from the initial decrease in absorbance for $\text{K}_3[\text{Fe}(\text{CN})_6]$ and expressed as μmol NADH used per min per mg membrane protein.

NADH oxidase (EC 1.6.99.3) activity. The membranes were treated in the same manner as for the NADH dehydrogenase measurements. Final concentrations were the same except that $\text{K}_3[\text{Fe}(\text{CN})_6]$ was omitted. The reaction was followed at 340 nm and 37°C . Activities were calculated from the absorbance decrease of NADH and expressed as μmol NADH oxidized/min per mg membrane protein.

Mg²⁺-dependent ATPase (EC 3.6.1.3). The assay mixture contained 40–240 μg membrane protein, 50 mM Tris-HCl (pH 8.0), 1.25 mM MgCl_2 , 1.1 mM NaCl, 0.6 mM 2-mercaptoethanol in a volume of 1.0 ml. The reaction was started by adding 10 μl 125 mM ATP and was permitted to proceed for 30 min at 37°C with agitation. Blanks and zero time samples were also run. The reaction was terminated by adding 1.0 ml 10% (w/v) trichloroacetic acid and cooling the test tubes. Precipitated protein was removed. Liberated inorganic phosphate (from ATP) was determined according to Chen et al. [25]. Enzyme activity was expressed as nmol phosphate liberated from ATP/min per mg membrane protein.

Electron microscopy

For negative contrasting 2% (w/v) potassium phosphotungstate (pH 7.0) was used. For thin sectioning a pellet of membranes was suspended in 3% (v/v) glutaraldehyde, pH 7.0, and left for 1 h at 20°C . The membranes were then washed with Michaelis buffer, pH 7.0, post-fixed with osmium tetroxide and uranyl acetate, as described by Ryter and Kellenberger [26], and embedded in Epon. Sections were stained with uranyl acetate for 1 h at 20°C .

Density gradient centrifugation

Linear 18 ml 30–60% (w/w) sucrose gradients in β -buffer diluted 1 : 20 were prepared. Membranes were centrifuged at $99\,000 \times g$ for 3.5 h at 3°C in a 3×20 ml swing-out rotor. The gradients were unloaded through a puncture in the bottom of the tubes. Absorbance at 280 nm was recorded.

For comparison membranes disintegrated by five consecutive passages through an X-press with intermittent freezing [27] were run on gradients under identical conditions.

Results

Countercurrent distribution

The phase system found suitable for the partition of *A. laidlawii* membranes (Materials and Methods) is one which has an electrostatic potential difference between the phases (top phase positive) [18]. Visual examination of membrane turbidity in the tubes of the countercurrent extraction train revealed that membranes with low partition coefficient were in the bottom phase, those with intermediate partition coefficients at the interphase, and those with high partition coefficients in the top phase. This visual inspection not only showed at once that membranes in different parts of the extraction were truly different one from another but also that factors in addition to charge are involved in determining the partition coefficient of these membranes since at least a portion of the negatively charged membranes are in the negatively charged bottom phase. Determination of membrane content in different tubes by measurement of absorbance at 500 nm indicated that the membranes were composed primarily of two subpopulations.

By selectively supplementing the growth media with various amounts of one saturated and one unsaturated fatty acid, i.e. palmitic and oleic acids, stepwise changes in the physical properties of the membranes were introduced.

In Fig. 1A the separation pattern of membranes from cells grown on 0.120 mM palmitic plus 0.030 mM oleic acid is shown.

Membranes from cells grown on 0.090 mM palmitic plus 0.060 mM oleic acid were also composed of two subpopulations (Fig. 1B). Here, the 'right-hand' subpopulation was the larger one.

A further increase of oleic acid in the growth medium at the expense of palmitic acid, i.e. 0.030 mM palmitic acid plus 0.120 mM oleic acid, caused the 'right-hand' subpopulation to further increase in quantity (Fig. 1C). The 'left-hand' subpopulation became smaller but was still distinguishable.

Ultrastructure

The morphology of *A. laidlawii* cells is very pleomorphic. Fig. 2, A, B and C shows the ultrastructure of unfractionated, 'left-hand' and 'right-hand' subpopulations, respectively, as seen in sectioned specimens. The size distribution of the unfractionated membranes indicates that a cell cluster gave rise to several individual membranes on osmotic lysis. Holes in some of the membranes were visible. These results were confirmed by observations on negatively-stained membranes. Membranes separated by countercurrent distribution seemed to consist of a larger number of small vesicles than did the unfrac-

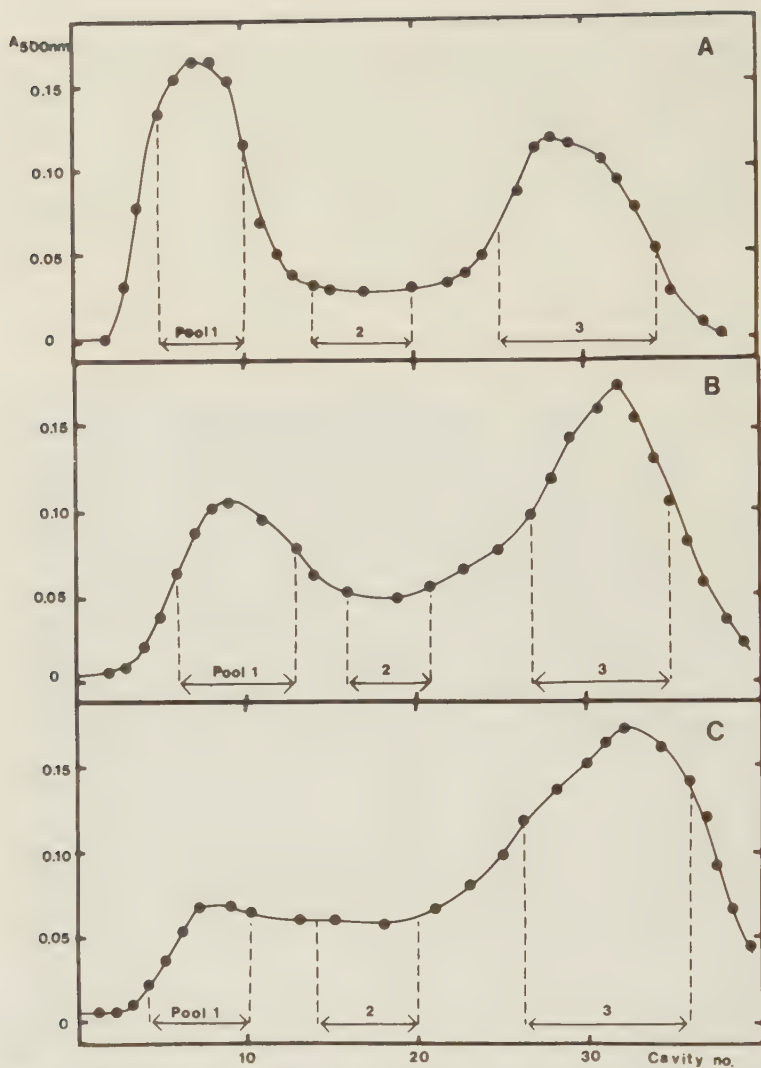


Fig. 1. Countercurrent distribution patterns of *A. laidlawii* membranes from cells grown with (A) 0.120 mM palmitic acid plus 0.030 mM oleic acid, (B) 0.090 mM palmitic acid plus 0.060 mM oleic acid and (C) 0.030 mM palmitic acid plus 0.120 mM oleic acid. Pools 1, 2 and 3: membranes within these fractions were pooled for chemical, enzymatic and ultrastructural analyses. Phase system contained 5% (w/w) dextran, 4% (w/w) poly(ethylene glycol), 8.3 mM sodium phosphate, pH 7.4, and 3 mM NaCl per kg final mixture. 39 Transfers were completed at 3°C.

tionated ones (Fig. 2, B and C). Many, but not all, vesicles appeared to be closed. No significant enrichment of membranes of a given size could be detected in either of the two subpopulations.

Lipid composition of different membrane fractions

Table I shows the lipid composition of the different pools as well as the saturation ratio of the lipids. Several features are evident. The quantity of each lipid is not the same in different pools from a separation series. The largest relative differences are in those lipids which comprise the minor components

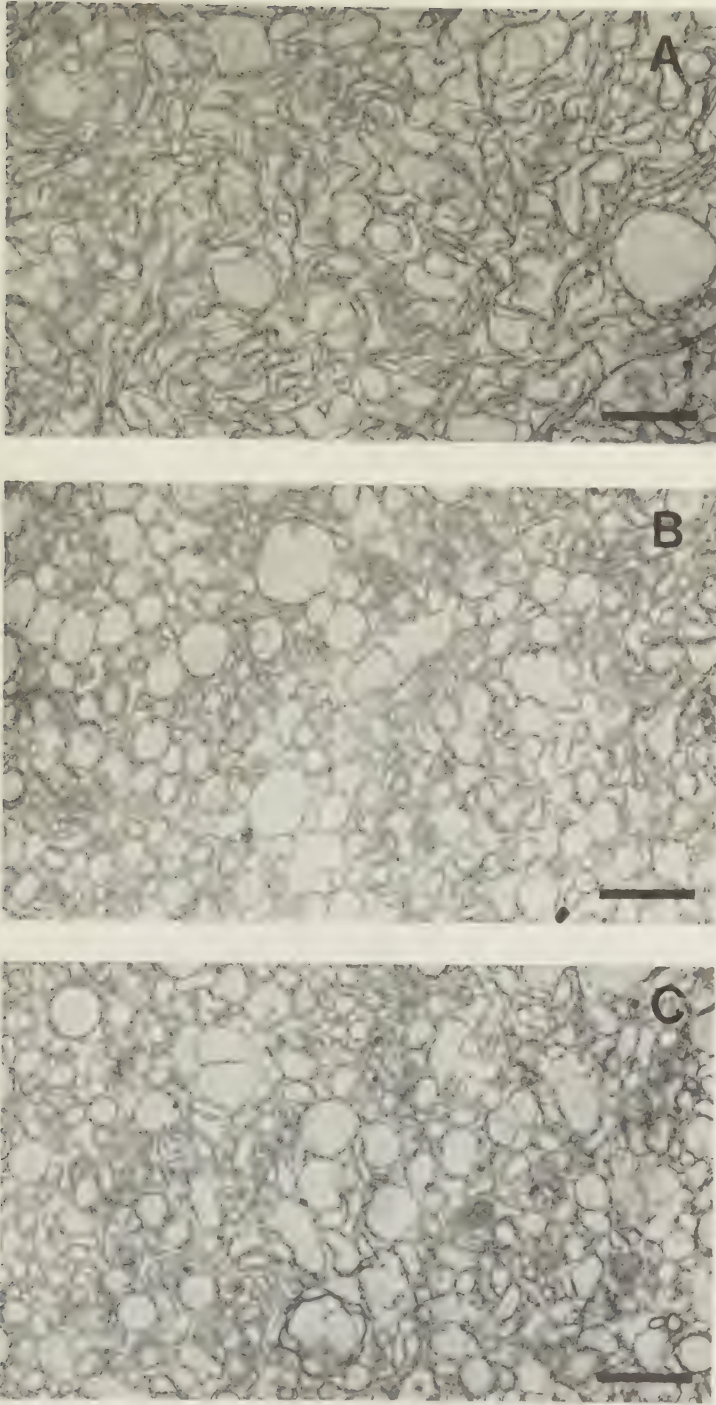


Fig. 2. Thin section electron micrographs of different membrane fractions obtained by countercurrent distribution. A, unfractionated membranes; B, membranes from a 'left-hand' population (see text); C, membranes from a 'right-hand' population. See Materials and Methods for technical data. The bars represent 1.0 μm .

TABLE I
LIPID COMPOSITION OF DIFFERENT MEMBRANE FRACTIONS FROM A. LAIDLAWII OBTAINED BY COUNTERCURRENT DISTRIBUTION
Pools 1, 2 and 3, see Figs. 1A, 1B and 1C for details. Lipid amounts in mol/100 mol.

Lipids	Fatty acids added in the growth medium								
	0.120 mM Palmitic acid 0.030 mM Oleic acid			0.090 mM Palmitic acid 0.060 mM Oleic acid			0.030 mM Palmitic acid 0.120 mM Oleic acid		
	Pool: 1	2	3	1	2	3	1	2	3
Charged lipids *	13.8	14.7	13.2	16.7	20.0	17.6	19.6	20.3	26.2
Diglucoxydiglyceride	18.2	18.0	22.1	31.2	28.0	34.3	41.1	42.0	41.7
Monoglucoxydiglyceride	45.5	45.4	46.2	39.1	38.2	35.1	31.4	10.1	26.7
Glucolipid X	15.7	16.9	13.2	9.6	8.7	9.2	5.7	25.3	3.9
Ratio Monoglucoxydiglyceride to Diglucoxydiglyceride (mol/mol)	2.52	2.52	2.09	1.25	1.36	1.02	0.76	0.24	0.64
Ratio nmol lipids/ μ g membrane protein	0.28	0.50	0.46	0.85	0.90	0.70	0.85	0.86	0.75
Ratio palmitic acid/oleic acid in lipids (mol/mol)	2.22	2.26	2.62	1.17	1.30	1.31	0.75	0.84	0.84

* Charged lipids: glycerophosphorylmono- and diglucoxydiglyceride plus phosphatidylglycerol (see ref. 8).

(data not shown), but the dissimilarities between the dominating glycolipids are larger on a total basis. The monoglucosyldiglyceride/diglucosyldiglyceride ratio varies considerable from pool to pool (Table I). Furthermore, the amounts of charged lipids, i.e. phosphatidylglycerol and the glycerophosphoglycolipids, are not constant. The lipid saturation ratios (although differing between individual lipids) all increase with increasing partition coefficients (i.e. to the right). As noted earlier [8] increased amounts of a saturated fatty acid in the growth medium, i.e. palmitic acid, result in increased monoglucosyldiglyceride/diglucosyldiglyceride ratio and more vigorous synthesis of the apolar glucolipid X.

Membrane protein composition

Sodium dodecyl sulphate polyacrylamide gel electrophoresis showed more than 40 protein bands (Fig. 3) with a molecular weight range from 14 300 to

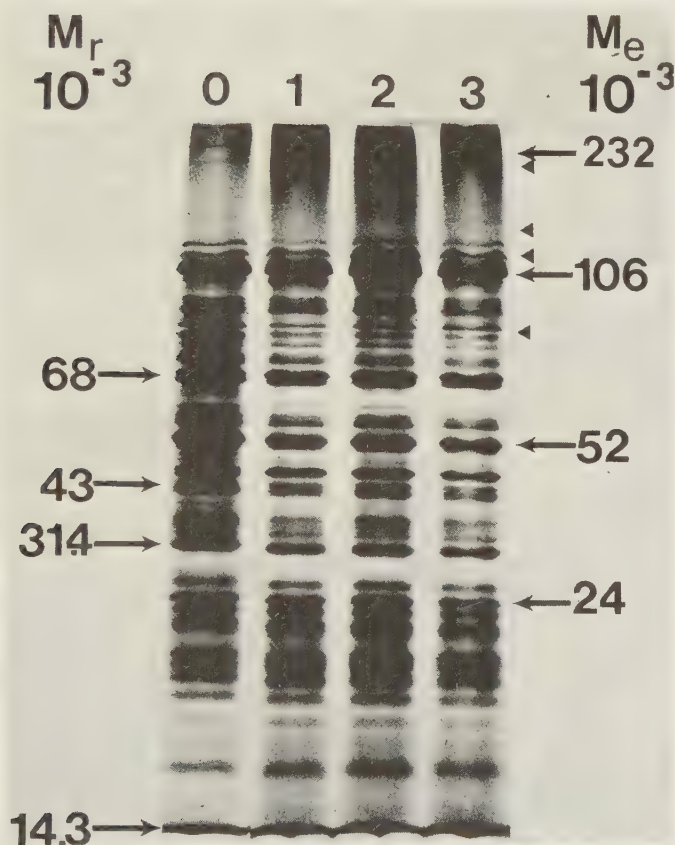


Fig. 3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of *A. laidlawii* proteins in different membrane fractions obtained by countercurrent distribution. The membranes were derived from cells grown on 0.120 mM palmitic acid plus 0.030 mM oleic acid. Sample 0, unfractionated membranes; samples 1, 2 and 3, membranes from countercurrent distribution pools 1, 2 and 3 (see Fig. 1). M_r , reference molecular weights; M_e , estimated molecular weights of *A. laidlawii* proteins; ▲, protein bands that differ between fractions.

230 000. The similarity between the unfractionated membranes and the pooled fractions was striking. Quantitative differences were noted in some bands in the high molecular weight range.

The protein patterns of unfractionated membranes showed quantitative variations in some bands probably as a consequent of differences in fatty acid supplementation in the growth medium (data not shown). The greatest difference was in protein having molecular weights in excess of 70 000.

Enzyme activities

The NADH dehydrogenase and oxidase activities were compared by digesting the membranes in an excess of sodium deoxycholate and the activities were estimated in the absence of polymers. Since the ATPase is very sensitive to detergent, this treatment was not used. Table II shows that both NADH dehydrogenase and oxidase activities differ significantly between unfractionated membranes and the different pools. The ratio between the two activities is also inconstant in the pools. NADH oxidase activities were always much lower in partitioned membranes than in unfractionated membranes. For NADH oxidase, highest specific activities in the partitioned membranes were always in fractions between the two main subpopulations.

In the case of the ATPase highest specific activities were found in the smallest subpopulations (cf. Figs. 1A, 1B and 1C). This enzyme also had significantly different activities in the various pools.

Influence of membrane cholesterol

Although the distribution of polar lipid heads varied among pools, fatty acid properties are probably important for maintaining such heterogeneity. Lipid interactions can also be modified by the insertion of cholesterol [28]. To make the interpretation more straightforward, cholesterol was introduced into membranes containing only one fatty acid, i.e. oleic acid [8]. Here, endogenous

TABLE II

ENZYME ACTIVITIES IN DIFFERENT MEMBRANE FRACTIONS

Added fatty acids	Pools	NADH dehydrogenase ($\mu\text{mol NADH}/\text{min per}$ $\text{mg membrane protein}$)	NADH oxidase ($\mu\text{mol NADH}/\text{min per mg mem-}$ brane protein)	ATPase ($\text{nmol ATP}/\text{min}$ per mg membrane protein)
0.120 mM Palmitic acid + 0.030 mM oleic acid	0 *	1.02	2.53	81
	1	1.76	0.64	57
	2	1.08	1.21	73
	3	0.72	1.12	122
0.090 mM Palmitic acid + 0.060 mM oleic acid	0 *	0.84	2.05	70
	1	0.97	0.81	145
	2	0.79	1.06	67
	3	1.15	0.40	51
0.030 mM Palmitic acid + 0.120 mM oleic acid	0 *	0.71	1.38	38
	1	1.03	0.79	46
	2	1.22	0.85	37
	3	0.99	0.79	24

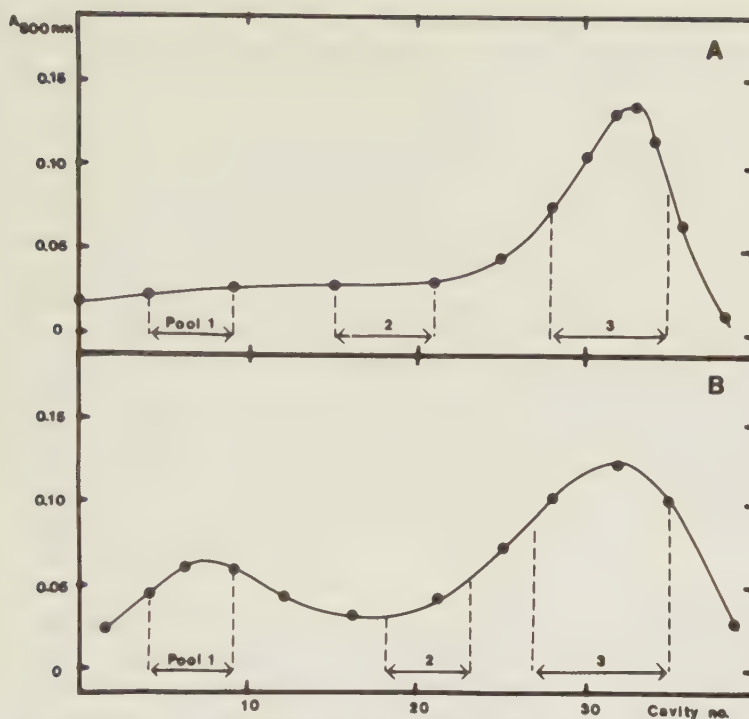


Fig. 4. Countercurrent distribution patterns of membranes from cells grown on (A) 0.150 mM oleic acid and (B) 0.150 mM oleic acid plus 0.020 mM cholesterol. See Fig. 1 for details.

TABLE III

LIPID AND CHOLESTEROL COMPOSITION OF DIFFERENT MEMBRANE FRACTIONS CONTAINING OLEIC ACID

Pools 1, 2 and 3, see Figs. 4A and 4B for details. Lipid amounts in mol/100 mol.

Lipids	Supplements					
	0.150 mM Oleic acid			0.150 mM Oleic acid plus 0.020 mM cholesterol		
	Pool: 1	2	3	1	2	3
Charged lipids *	34.6	35.3	36.1	43.0	49.1	43.3
Diglucoacyldiglyceride	34.6	36.2	38.5	34.4	36.7	40.5
Monoglucoacyldiglyceride	23.8	22.7	22.9	13.5	9.1	8.4
Ratio monoglucoacyldiglyceride/ diglucoacyldiglyceride (mol/mol)	0.69	0.63	0.59	0.39	0.25	0.21
Ratio nmol lipids/ μ g membrane protein	0.38	0.58	0.67	0.20	0.29	0.31
Ratio cholesterol/total lipids (mol/mol)	—	—	—	0.19 **	0.48	0.33

* Charged lipids: glycerophosphorylmono- and diglucoacyldiglyceride plus phosphatidylglycerol (see ref. 8).

** Ratio in unfractionated membranes, 0.29.

saturated fatty acid synthesis is very low [8]. Fig. 4A shows the distribution pattern obtained from membranes containing oleic acid alone. In keeping with trends evident in Fig. 1 A, B and C, the 'left-hand' population completely vanished in the absence of a saturated fatty acid.

Incorporation of cholesterol (during growth) in these membranes caused the 'left-hand' population to reappear (Fig. 4B). Lipid analysis disclosed a more homogeneous distribution of lipids between pools when cholesterol was absent (Table III). The monoglucosyldiglyceride/diglucosyldiglyceride ratio also differed more in membrane pools containing cholesterol. The molar ratio of cholesterol to total lipids varied almost 2-fold between the two subpopulations compared to the average value which was 0.29 in unfractionated membranes (Table III). The 'left-hand' subpopulation contained more monoglucosyldiglyceride and less diglucosyldiglyceride, phosphatidylglycerol and cholesterol than unfractionated membranes. The opposite was true for the 'right-hand' subpopulation. Membranes from cavities between these two main subpopulations (i.e. pool 2) had significantly more charged lipids and cholesterol.

Discussion

The conditions used cause *A. laidlawii* to grow in large filamentous cell clusters surrounded by one common cell membrane. Comparisons of normal [29] and thick [30] electron microscopic sections of *A. laidlawii* cell clusters with the membranes obtained here (Fig. 2A) indicate that, on the basis of size, each cell cluster gives rise to several membranes on lysis. These membranes yield a homogeneous peak when centrifuged on a sucrose gradient (data not shown). When membranes were broken in an X-press several subpopulations were obtained. These cannot be considered natural since drastic pressure-collapse treatments are needed to produce them. Furthermore, a significant amount of protein is lost (data not shown). Several enzymes lost their activities and such membranes could not be properly separated by countercurrent distribution due to clumping.

It is thus evident that membranes from *A. laidlawii*, which are homogeneous with respect to buoyant density, can be separated on the basis of their surface properties into (at least) two subpopulations (Figs. 1A, B and C and 4B). Analysis of membrane proteins in different subpopulations reveals very small quantitative differences. Since these occur among the higher molecular weight proteins the total number of proteins differing is small. In contrast to other observed differences (see below), we consider these specific proteins to be of minor importance in determining the membrane partition coefficient.

Since the two-polymer aqueous phase system reflects membrane surface properties [18,21], the ratio between protein and lipids may be of importance. This ratio will, to some extent, determine the exposure of charged and/or hydrophobic surface groups. Examination of ratios obtained with different *A. laidlawii* membranes (Tables I and III) shows large fluctuations which appear not directly related to the partition coefficients of the two subpopulations (Figs. 1A, B and C and 4B).

Lipid composition and saturation ratios (i.e. palmitic/oleic acid) have a direct bearing on both the appearance and relative sizes of membrane subpopu-

lations. In *A. laidlawii* A membranes the physiological ratio between the two dominating glycolipids, monoglucosyldiglyceride and diglucosyldiglyceride, undergoes profound variations in response to changes in membrane viscosity caused by changes in fatty acid content [8], temperature and cholesterol content [9]. NMR and X-ray studies revealed great variations in physical properties between these lipids [10], the most important being that monoglucosyldiglyceride has a small upright polar head while the polar head of diglucosyldiglyceride has an alignment parallel to the bilayer plane and is only capable of binding a limited amount of water [10]. The exposure of the hydrophilic parts of the membrane polar heads is, thus, probably greater in those membranes which are rich in monoglucosyldiglyceride. In an analogous argument, this exposure will probably be less when diglucosyldiglyceride occurs in large amounts because of interference by its polar head. Some recent findings substantiate these structural differences, e.g. that monoglucosyldiglyceride in monolayers is less sensitive to increases in surface pressures when reacting with concanavalin A than is diglucosyldiglyceride [31]. Furthermore, a certain excess of monoglucosyldiglyceride over diglucosyldiglyceride makes the cell membrane of an *A. laidlawii* mutant resistant to reactive complement lysis [32].

With decreasing amounts of palmitic acid in the membrane lipids during growth, decreasing amounts of monoglucosyldiglyceride are synthesized (Tables I and III) in agreement with an earlier proposed mechanism [8]. A large ratio of monoglucosyldiglyceride to diglucosyldiglyceride (Table I) correlates positively with a large 'left-hand' subpopulation (Figs. 1A, B and C). Below certain lipid ratios (Table III) the 'left-hand' population disappears (Fig. 4A). Since cholesterol, being able to interact sterically with the lipid acyl chains, restores the 'left-hand' population at a lipid ratio below the critical one, lipid steric interactions are most probably involved. The phase system used appears to separate *A. laidlawii* membranes both on the basis of charge and hydrophobic/hydrophilic properties. The positively charged top phase should interact with negatively charged *A. laidlawii* membranes. However, in membranes in which hydrophilic surface groups are pronounced, these may predominate in determining the partition coefficient obtained. The 'left-hand' population was primarily in the bottom phase. A large 'left-hand' population thus means that a substantial part of the membranes partitioned due to surface properties other than charge. These (probably hydrophilic) properties manifest themselves because of the polar head configuration of the dominating monoglycosyldiglyceride. Other aspects that may influence the membrane partition behaviour include the contribution of glucolipid X [8] and the consistently larger fraction of oleic acid in the lipids in a 'left-hand' population than in the corresponding 'right-hand' peak (Table I).

In addition to decreased glycolipid ratios, membranes having only oleic acid contain a larger fraction of the negatively charged lipids. Both of these properties will increase the 'right-hand' population, which is thus separated primarily because of the affinity between negatively-charged membranes and positively-charged top phase. That cholesterol can 'induce' the appearance of a 'left-hand' population (Figs. 4A and 4B), despite the very high amounts of charged lipids in these membranes (Table III), is noteworthy. Cholesterol content in the 'left-

hand' population is substantially lower, and the concentration in the 'right-hand' population higher, than the mean (Table III). Membranes partitioned between these main populations contain more charged lipids and cholesterol than average. These results indicate that specific lipid-cholesterol interactions occur. Furthermore, exposure of charged groups may also be influenced by the presence of cholesterol.

The difference in specific activities of enzymes in the subpopulations further strengthens the concepts of an heterogeneous membrane organisation. Since the molecular weights for the enzymes tested are not known, it is not possible to relate the different activities with differences in the membrane protein patterns. The use of deoxycholate in the NADH dehydrogenase and oxidase assays rules out the possibility that different sidedness of the membranes is responsible for the observed differences. Neither of these enzymes is dependent on lipid, but several workers have pointed to the importance of microenvironment on them [33,34]. Clearly, different lipid organization may alter their functions. The obtained activities may also be a result of a difference in membrane age since the enzymes are known to vary with age in growing cells [34]. ATPase has recently been shown to depend on phosphatidylglycerol for maximum activity [4]. Although a small amount of the total phosphatidylglycerol molecules was needed, different organization of phosphatidylglycerol in the membrane subpopulations could affect the activity of the enzyme.

Since the enzymes tested are all important mediators of the energy-yielding mechanisms in *A. laidlawii*, their different activities in the subpopulations might mirror the status of energy metabolism in these membranes.

In summary, we conclude that *A. laidlawii* membranes, considered homogeneous by standard criteria, consist of at least two subpopulations under normal conditions with regard to surface properties. These subpopulations differ in their polar lipid content, lipid/protein ratios, enzyme activities and, to some extent, in fatty acid content. The latter components seem to be of importance in maintaining the heterogeneity. Such variations in surface properties will most likely affect the relationship between the parasite *A. laidlawii* and its hosts.

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Lipid Bilayer Stability in Membranes. Regulation of Lipid Composition in *Acholeplasma laidlawii* As Governed by Molecular Shape[†]

Åke Wieslander,* Anders Christiansson, Leif Rilfors, and Göran Lindblom

ABSTRACT: The polar lipid composition in membranes of *Acholeplasma laidlawii* is extensively regulated as a response to environmental changes. In particular, the ratio between the dominating lipids monoglucosyldiglyceride and diglucosyldiglyceride is altered depending on temperature, configuration of incorporated fatty acids, and membrane cholesterol content. Synthesis of monoglucosyldiglyceride is stimulated by low temperature and saturated fatty acids but diminished by the presence of cholesterol. These factors are likely to affect the molecular geometry of the membrane lipids. Monoglucosyldiglyceride and diglucosyldiglyceride have wedge- and rodlike molecular shapes, respectively, that are modifiable to a certain extent. The packing constraints of lipids in amphiphilic aggregates, i.e., hydrocarbon-water interfacial area, hydrocarbon chain volume, and hydrocarbon chain length, are very important in determining the aggregate

structure [Israelachvili, J. N., Mitchell, D. J., & Ninham, B. W. (1976) *J. Chem. Soc., Faraday Trans. 2* 72, 1525]. Pure monoglucosyldiglyceride forms a reversed hexagonal (H_{II}) phase structure with different fatty acid contents, while diglucosyldiglyceride forms a lamellar phase. However, the only lipid structure compatible with a functional biological membrane is the lamellar phase. Consequently, the balance between lipids forming lamellar and other mesophase structures must keep within certain limits. Here we show that the response in *A. laidlawii* lipid metabolism following external and internal stimuli can be predicted on the basis of molecular shapes and is necessary for the cell in order to maintain optimal membrane stability. Furthermore, the reduced capacity of *Acholeplasma* membranes to incorporate cholesterol is another consequence of this regulation, aiming at preservation of bilayer stability.

The functional state of biological membranes involves a dynamic cooperation between the physiologically active proteins and the lipid matrix. Most physicochemical investigations of these membranes have focused on the gel to liquid crystalline phase transition and its influence on lipid lateral packing (Engelman, 1971), growth temperature adaptation (McElhaney, 1974), permeability characteristics of water, ions, and

other species (Razin, 1975a), enzyme and transport activities (Read & McElhaney, 1975; Silvius et al., 1978), and protein disposition (Verkleij, 1975).

Concerning the contribution of lipid polar head groups to membrane function and stability, little is known. However, the maintenance of a critical balance between lipids with different anionic polar head groups has been observed in *Escherichia coli* (Raetz, 1978). Recent findings also show that the activities of a very limited number of membrane-associated enzymes are dependent on a specific lipid surrounding (Sandermann, 1978).

Our previous investigations have focused on the relationships between physiological regulation mechanisms and physical

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properties of membrane lipids from the wall-less procaryote *Acholeplasma laidlawii* strain A (Carlemalm & Wieslander, 1975; Wieslander & Rilfors, 1977; Christiansson & Wieslander, 1978, 1980; Wieslander et al., 1978, 1979). Three distinct ways for regulating the membrane lipid composition have been discovered in this strain: (1) incorporation of fatty acids into membrane lipids is governed by growth temperature as well as the presence of cholesterol; (2) the relative syntheses of the dominating polar lipids monoglucosyldiglyceride (MGDG)¹ and diglucosyldiglyceride (DGDG) are related to each other; they are influenced by changes in the apparent molecular ordering of the hydrocarbon chains caused by growth temperature, fatty acid content, and the presence of cholesterol; (3) the total balance between ionic and nonionic lipids seems to depend on the average lateral packing area occupied by the lipid molecules in the membrane. Such drastic changes in polar head group composition and in the ratio ionic/nonionic lipids have not been observed in other membranes, cf. *E. coli* wild type (Raetz, 1978). Investigations on lipid packing properties in the dry crystalline state (Carlemalm & Wieslander, 1975; Carlemalm, 1976), water binding capacities, transition temperatures, phase structures (Wieslander et al., 1978), and lateral diffusion (Å. Wieslander, L. Rilfors, L. B.-Å. Johansson, and G. Lindblom, unpublished results) of membrane lipids from this *A. laidlawii* strain have also been performed.

In this paper we will show how the physical properties of the membrane lipids of *A. laidlawii* can be related to membrane stability. The only lipid structure compatible with a functional biological membrane is the lamellar one. However, it has been found that often this phase is not the thermodynamically stable one formed by a single membrane lipid-water system at physiological temperatures. Typical membrane lipids forming hexagonal mesophases are phosphatidylethanolamine (PE) (Cullis & De Kruijff, 1978b), diphosphatidylglycerol (DPG) (Rand & Sengupta, 1972), monogalactosyldiglyceride (Shipley, 1973), MGDG (Wieslander et al., 1978), and sphingomyelin (Yeagle et al., 1978). Mixtures of MGDG and DGDG can form a cubic mesophase (Å. Wieslander, L. Rilfors, L. B.-Å. Johansson, and G. Lindblom, unpublished results).

The tendencies for amphiphiles to form different structures (micelles and lamellar or hexagonal phases) can be understood from a unified theory that links interaction free energy, molecular geometry, and thermodynamics (Israelachvili et al., 1976, 1977). Two opposing forces contribute to the interaction free energy. The attractive interactions are the hydrophobic effect (Tanford, 1973), which tends to eliminate the contact between water and the hydrophobic core. Repulsive interactions arise from electrostatic and steric head group repulsion. Steric acyl chain repulsion is also involved. These interactions aim at keeping the total interaction free energy per lipid molecule at a minimum, resulting in an optimal surface area per head group, i.e., the area occupied by the polar head at the hydrocarbon-water interface. Thus, the packing constraints of the lipids in the amphiphilic aggregate are important factors in determining the aggregate shape (Israelachvili et al., 1976, 1977). These authors derived geometric expressions relating the hydrocarbon-water interfacial area a , the hydrocarbon chain volume v , and the hydrocarbon chain length l to the aggregate structure. The following results were ob-

tained for various aggregate shapes: spherical micelle, $v/al = 1/3$; rodlike micelle, $v/al = 1/2$; lamellae, $v/al = 1$. It follows that v/al must be larger than 1 for an aggregate building up a reversed hexagonal (H_{II}) phase, i.e., water cylinders in a hydrocarbon matrix (Ekwall, 1975).

Recently, Wennerström (1979) showed that the theory by Israelachvili et al. is of great importance for the understanding of the phase diagrams of soap-water systems. He concludes that the formation of a lamellar phase on addition of alcohol to a micellar solution or a hexagonal (H_I) phase is due to the fact that the hydrocarbon volume v is enhanced, i.e., v/al approaches unity. Similarly, the lipid organization in a bilayer is a delicate balance between various packing constraints, which determine the membrane stability. All other mesophase structures but the lamellar phase are prohibited. Thus, in a multicomponent bilayer system the geometrical properties of the individual lipid molecules are very important factors. In this work we apply the self-assembly theory by Israelachvili et al. to explain the observed physiological regulation of the different major lipids in *A. laidlawii*. It is found that the response in lipid metabolism following external and internal stimuli can be predicted by the theory.

Results and Discussion

Synthesis and Physical Properties of Membrane Lipids. *A. laidlawii* has an obligate demand for unsaturated fatty acids that it cannot synthesize (Pollack & Tourtellotte, 1967). Saturated fatty acids are synthesized by the malonyl-coenzyme A pathway (Rottem & Panos, 1970). This synthesis is inhibited by exhaustion of pantothenic acid, a precursor to coenzyme A and acyl carrier protein, from the growth medium (Christiansson & Wieslander, 1980). An absolute control of fatty acid incorporation from exogenous sources can thus be achieved (Wieslander & Rilfors, 1977; Christiansson & Wieslander, 1978, 1980).

In *A. laidlawii* strain A (EF22) the following polar lipids occur (Wieslander & Rilfors, 1977): MGDG, DGDG, an apolar monoglycerol, phosphatidylglycerol (PG), and glycerophosphoryl derivatives of MGDG and DGDG. Under certain growth conditions DPG is synthesized (Christiansson & Wieslander, 1978). Although uncharged at physiological pH, the glucolipids are cationic selective (Hopper et al., 1970) and calcium ion binding (Wieslander et al., 1978) in resemblance to PE. PG, DPG, and the phosphoglycerolipids each carry one negative charge. The tentative lipid biosynthetic pathways are shown in Figure 1.

²H NMR on heavy water and ²H-labeled acyl chains together with low-angle X-ray diffraction for lipid-water samples reveal a reversed hexagonal (H_{II}) phase structure for MGDG with different fatty acid contents, whereas DGDG and the other lipids form lamellar phases (Wieslander et al., 1978). DPG can alter between the two phase structures (Rand & Sengupta, 1972). Maximum hydration (7–9 mol of H₂O/polar head group) and phase transition temperatures for MGDG and DGDG are very similar to those of both synthetic and naturally occurring PE (Wieslander et al., 1978). The transition temperatures are about 20 °C above that of phosphatidylcholine with the corresponding fatty acid content (Wieslander et al., 1978).

Lipid Molecular Shape. The various lipid molecules in the bilayer can be visualized as building blocks with different geometries (Figure 2). The hydrocarbon chain length, hydrophobic volume, and polar head group area will determine the overall shape of the blocks (see also introductory statement). The phase structure formed by the different lipids is determined by this molecular shape.

¹ Abbreviations used: MGDG, monoglucosyldiglyceride; DGDG, diglucosyldiglyceride; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; NMR, nuclear magnetic resonance.

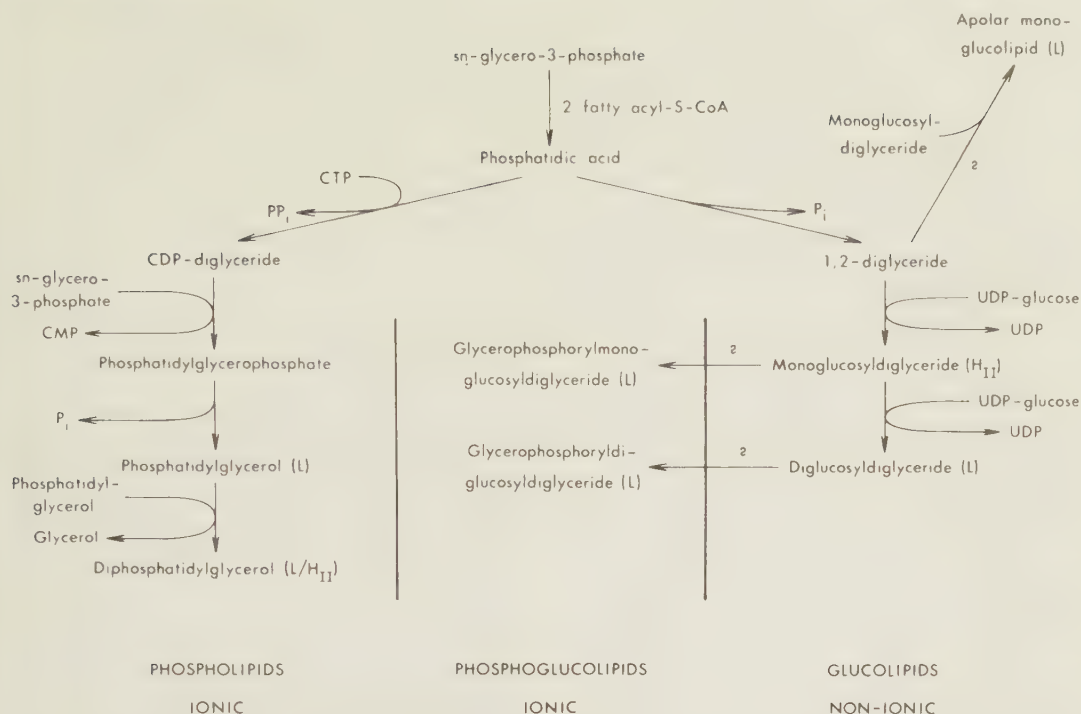


FIGURE 1: Tentative pathways for membrane polar lipid biosynthesis in *Acholeplasma laidlawii* strain A. Abbreviations used: CMP, cytidine 5'-monophosphate; CDP, cytidine 5'-diphosphate; CTP, cytidine 5'-triphosphate; UDP, uridine 5'-diphosphate; P_i, inorganic orthophosphate; PP_i, inorganic pyrophosphate; CoA, coenzyme A; L, lamellar phase structure; H_{II}, reversed hexagonal phase structure; ?, suggested pathway.

(i) With a given *fatty acid composition*, i.e., constant values of *v* and *l*, any change in the size (bulkiness, charge, hydration) of the polar head group will affect the optimal surface area *a* of the lipid molecule. Modifications of polar head structure can thus alter the packing properties (*v/a*) of an individual lipid in the bilayer (Figure 2A).

(ii) For lipids with a given *polar head group*, i.e., constant value of *a*, the molecular shape depends on several factors. A substitution of unsaturated fatty acids (Figure 2B, left) for saturated ones, especially with *cis* double bonds (Figure 2B, right), will reduce the length of the hydrocarbon chains (*l*) and increase the width of the hydrocarbon space almost without any change in the hydrophobic volume (*v*) (Träuble & Haynes, 1971; Israelachvili et al., 1977). By increasing the temperature, *trans-gauche* isomerizations and 2g1 kinks are introduced into the hydrocarbon chains (Mendelsohn & Taraschi, 1978; Träuble & Haynes, 1971), yielding the same effects as mentioned above (Figure 2B).

(iii) *Cholesterol* has a pronounced wedge shape (Carnie et al., 1979; Figure 2B). Furthermore, cholesterol slightly diminishes the lateral packing area of an individual lipid in a monolayer, since the observed mean molecular areas for cholesterol-lipid mixtures are smaller than those obtained by theoretical calculations (Demel & De Kruijff, 1976). However, the presence of cholesterol will nevertheless largely increase the distances between all the other lipid molecules.

Lipid Regulating Mechanisms in *Acholeplasma laidlawii*.

(A) *Fatty Acid Incorporation.* The potential capacity of *A. laidlawii* to regulate membrane fatty acid incorporation is best evoked by applying a temperature-shift technique (Christiansson & Wieslander, 1978). Cells grown in an equimolar mixture of palmitic and oleic acid incorporate more oleic acid

when shifted to 17 °C than cells maintained at 37 °C. This increase of oleic acid takes different courses with respect to different lipid species and time after the temperature decrease. The most rapid response occurs in MGDG, the polar lipid having the highest content of palmitic acid. In PG the oleic acid content rises after an initial lag period, whereas DGDG remains nearly unchanged. Thus, in order to maintain an optimal packing of the various lipids in the bilayer, according to the self-assembly theory, the cell can "afford" to incorporate unsaturated fatty acids into MGDG, although this lipid forms a reversed hexagonal (H_{II}) phase. In fact, since the temperature is lowered, it can be expected that the MGDG content is responsible for about the same packing constraint at the low temperature as it was at the higher temperature with more saturated fatty acids (Figure 2B), giving an optimal packing in the bilayer.

(B) *Regulation of Polar Head Group Composition.* Three different ways to change the polar head group composition have been exploited in *A. laidlawii* strain A (Wieslander & Rilfors, 1977; Christiansson & Wieslander, 1978, 1980): (i) variations in structure of incorporated *fatty acids*; (ii) changes in growth *temperature*; (iii) incorporation of *cholesterol*. The predominant response in polar lipid metabolism is a large change in the ratio between the structurally related glucolipids MGDG and DGDG (Figure 1). Furthermore, the ionic lipid fraction varies profoundly. These changes in *A. laidlawii* play a central role concerning the relation between lipid molecular shape and membrane bilayer stability.

(i) Successive changes in the hydrophobic geometry of the lipids caused by different incorporated ratios of saturated/unsaturated *fatty acids* yield correlated responses in the molar ratio MGDG/DGDG (Table I). Increased amounts of *cis*-

Table I: Polar Lipid Composition in Membranes from *A. laidlawii* A Grown with Different Amounts of Saturated and Unsaturated Fatty Acids (18 h, 37 °C)

	supplementation to the growth medium (μM palmitic/ μM oleic acid)					
	120/30	90/60	75/75	30/120	0/150	0/150 + 20 μM cholesterol ^a
% oleic acid in lipids (mol/mol)	30.3	44.4	49.6	55.2	95	95
ratio MGDG/DGDG	2.37	1.21	0.80	0.75	0.57	0.27
% ionic lipids of total (mol/mol)	13.5	17.2	22.9	25.5	35.9	43.1

^a The ratio cholesterol/total lipids is 0.29 (mol/mol), corresponding to about 15% (wt/wt), which is the maximum incorporable amount in *A. laidlawii*.

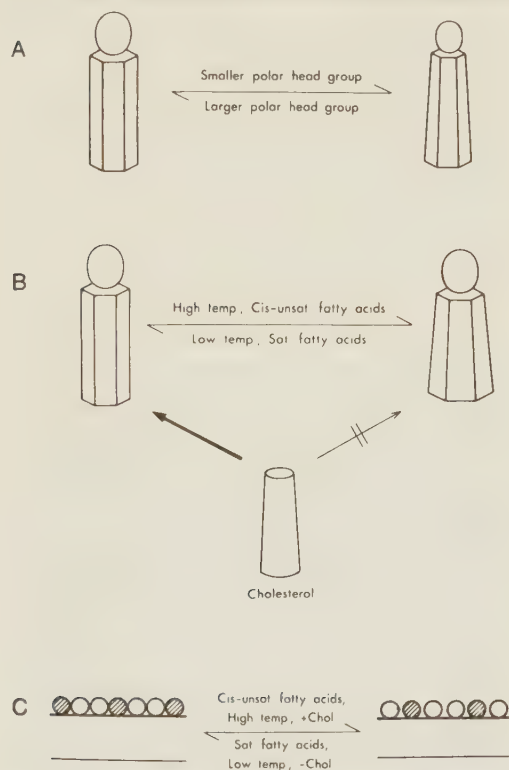


FIGURE 2: Molecular shapes of lipids. The geometrical properties of the lipid molecules determine their ability to pack together into a bilayer structure. (A) Molecules with identical fatty acid compositions. Left: geometry of lipids having a moderately large polar head group and a tendency to form a lamellar phase structure. Right: geometry of lipids having a small polar head group and thus a tendency to form a reversed hexagonal (H_{II}) phase structure due to the reduced hydrocarbon-water interfacial area. Note the pronounced wedge shape of the molecule. (B) Molecules with identical polar head groups. Left: geometry of lipids at temperatures below the phase transition temperature (T_i) and/or with saturated fatty acids only. Right: geometry of lipids at temperatures above T_i and/or containing cis unsaturated fatty acids. The shortening and broadening of the hydrophobic part of the molecule result in an increased tendency to form a reversed hexagonal (H_{II}) phase structure. Lower middle: molecular geometry of cholesterol. The arrows denote the possibilities for cholesterol to copack with lipids having different geometries and still preserve a stable bilayer structure. The dimensions of the molecules are based on literature values. (C) Membrane surface charge density. Left: polar head group density at temperatures below T_i and/or with lipids containing saturated fatty acids only. Right: polar head group density at temperatures above T_i and/or with lipids containing cis unsaturated fatty acids and/or cholesterol. Hatched circles denote anionic lipid molecules.

unsaturated fatty acids will increase the hydrophobic bulkiness of the lipid molecules (Figure 2B), leading to a more accentuated wedge shape, especially of MGDG, which fits better in a curved aggregate than in a bilayer structure. Consequently, the membrane stability is maintained by decreasing the ratio MGDG/DGDG upon an increase of cis unsaturation. The extent of variation of MGDG is 60% larger than that of DGDG, indicating that MGDG is the most actively regulated component. Similar alterations in the ratio MGDG/DGDG occur when the endogenous saturated fatty acid synthesis is stimulated by stepwise additions of the coenzyme precursor pantotheine (Christiansson & Wieslander, 1980).

By decreasing the molar fraction of cis-unsaturated fatty acid in the membrane from 95 to 30%, the ionic lipid fraction is reduced by almost a factor of three (Table I). This might be due partly to a compensation for the enhanced surface charge density caused by the decreased lateral packing area of the lipid (Figure 2C). Since the presence of divalent cations in the culture medium raises the gel to liquid crystalline transition temperature for the ionic lipids (Träuble, 1977), a reduction in the amount of these lipids may also be necessary to prevent the molecular ordering of the acyl chains from becoming too high.

(ii) A decrease in temperature leads to an increase in molecular order of the acyl chains, diminishing the hydrophobic bulkiness of the lipids (Figure 2). The magnitude of this effect depends on the existing fatty acid composition before the temperature shift (Figure 2B). Most biological membranes contain a certain fraction of wedge-shaped polar lipids. The presence of these lipids is of importance for bilayer stability, and, consequently, a decrease in temperature must be neutralized by accentuating the wedge shape properties. This can be achieved in two ways: (1) an enhanced incorporation of unsaturated fatty acids (section i above) and (2) increased synthesis of lipids with a small polar head group like MGDG (Figure 2A). The latter effect is demonstrated in Figure 3, where it is shown how the ratio MGDG/DGDG is altered with cell growth after a temperature shift and/or with different membrane fatty acid compositions. At the time of shift-down (12 h), the ratio of MGDG/DGDG differs significantly depending on the difference in fatty acid composition as shown in Table I. With both fatty acid supplementations this ratio is diminished during growth at 37 °C (Wieslander & Rillfors, 1977; Christiansson & Wieslander, 1978). However, at 17 °C the ratio rises due to a largely enhanced synthesis of MGDG (Christiansson & Wieslander, 1978). This effect is observed also when *A. laidlawii* is grown at a constant low temperature, 10 °C. Because of the shape of the molecule, MGDG will reestablish an optimal packing of the membrane components in the bilayer.

When passing the phase transition interval a decrease in temperature will decrease the lateral packing area of the lipids

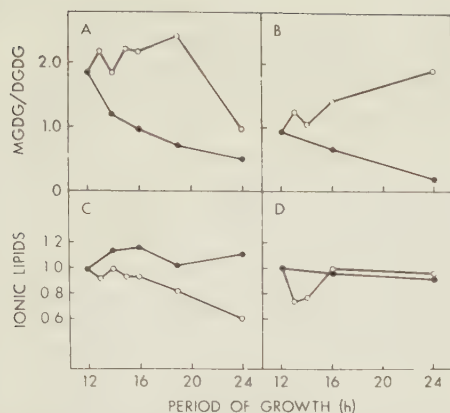


FIGURE 3: Regulation of polar lipid composition in *A. laidlawii* after a temperature shift: (A and B) ratio MGDG(H_{II})/DGDG(L); (C and D) relative amount of ionic lipids; the amount at 12 h is assigned the value of 1.0; (A, C) cells grown with 75 μ M palmitic acid and 75 μ M oleic acid; (B, D) cells grown with 150 μ M oleic acid; (●) growth at 37 °C; (○) cells shifted to 17 °C after growth for 12 h at 37 °C. The experimental conditions are described by Christiansson & Wieslander (1978).

and consequently increase the surface charge density (Träuble, 1977; Träuble & Haynes, 1971; Figure 2C). With membranes containing palmitic and oleic acid, a shift from 37 °C to a temperature within the phase-transition interval, e.g., 17 °C (Christiansson & Wieslander, 1978; Wieslander et al., 1978) yields a strong reduction in the biosynthesis of ionic lipids (Figure 3C). The reasons for this may be the same as mentioned in section i. With membranes containing oleic acid only, 17 °C is well above the phase-transition interval (cf. Figures 3C and 3D).

(iii) Addition of *cholesterol* to an *A. laidlawii* culture grown with an equimolar mixture of palmitic and oleic acid yields higher incorporation of oleic acid both at 37 °C and after a shift to 17 °C (Christiansson & Wieslander, 1978). Obviously cholesterol causes an increase in the ordering of the fatty acyl chains resulting in a more rodlike type of molecular geometry of the surrounding lipids. This is counteracted by an increased incorporation of unsaturated fatty acids in all lipids. Furthermore, since cholesterol has a significant wedge shape (Carnie et al., 1979; Figure 2B), insertion of this molecule into the membrane destabilizes the bilayer structure, especially in the presence of lipids forming a reversed hexagonal (H_{II}) phase. In *A. laidlawii* the formation of such a phase in the membrane is prevented by a relative reduction of the amount of MGDG by 40% (Table I). Incorporation of cholesterol into a membrane also dilutes the surface charge density (Figure 2C). *A. laidlawii* compensates for this by increasing the ionic lipid fraction (Table I).

Thus, according to the presented theory, an increase in the number of cis double bonds, an increase in temperature, as well as the presence of cholesterol destabilize the lamellar phase structure, which the cell must compensate for. This indeed occurs by a change in the polar head composition: a lowering of the MGDG lipid content and an increase in the amount of ionic lipids.

Concluding Remarks

It has been shown that for most purposes a qualitative application of the theory developed by Israelachvili et al. (1976, 1977) can be used for a sufficiently good description of the lipid bilayer stability in *Acholeplasma laidlawii* membranes.

Thus, it can be concluded that the molecular geometry of the lipids is of vital importance for the stability. Although the theory has been applied to the membrane of *A. laidlawii*, it can nevertheless be expected to hold also for other biological membranes. For example, it has been found that *E. coli* counteracts the effect of a drop in the growth temperature by an increase in the degree of cis unsaturation in the lipids (Cronan & Gelmann, 1975), which changes the geometry of the dominating polar lipid PE to a more pronounced wedge shape (Cullis & De Kruijff, 1978a). In *Bacillus megaterium* this temperature adaptation is accomplished by regulation of the fatty acid chain length and by the ratio between two different types of branched fatty acids (Rilfors et al., 1978), which influence the hydrophobic volume.

A proposal can be made to explain the occurrence of several different polar lipid species in the membranes of most organisms, and why their relative amounts must be actively regulated. These membrane properties most certainly would have been lost during evolution were they not of crucial importance for survival to the cell. An optimal packing of the lipid molecules is essential for obtaining a stable bilayer structure. This demand cannot be fulfilled by a single lipid species; it necessitates a mixture of lipids having tendencies to form lamellar and other mesophase structures (e.g., hexagonal, cubic). As shown in this work, the balance between such lipids must keep within certain limits, and this probably explains why some lipid species are not coexisting in biological membranes (Demel et al., 1977; Carnie et al., 1979). Furthermore, it has been proposed that local regions of lipids forming transitory nonlamellar phases may be advantageous to some membrane functions such as movement of membrane components between bilayer halves, fusion, exo- and endocytosis, and facilitated transport (Cullis & Verkleij, 1979). Nonlamellar phases have been reported for microsomal membranes (De Kruijff et al., 1978; Stier et al., 1978). It has also been suggested that packing constraints impose a structural coupling between membrane proteins and their surrounding lipids, and thus a heterogeneous composition of lipids with different molecular geometries may be required in order to minimize packing defects around the irregular surfaces of proteins (Israelachvili, 1977; Sandermann, 1978). This suggestion is strongly supported by protein-lipid interaction studies performed on *A. laidlawii* (K.-E. Johansson, C. Jägersten, A. Christiansson, and Å. Wieslander, unpublished experiments).

The balance between ionic and nonionic lipids is actively regulated in *A. laidlawii* membranes (Table I). The great capability of *A. laidlawii*, compared to *E. coli*, to regulate the ionic lipid fraction in response to environmental changes seems logical considering that the *A. laidlawii* membrane directly faces the surrounding physical milieu, while the *E. coli* membrane is enclosed by a cell envelope.

Our results also show the significance of knowing the phase diagram of the individual as well as mixed compositions of the various membrane lipids. Hitherto, few such diagrams have been reported in the literature. Recently, we discovered a cubic phase consisting of a mixture of MGDG and DGDG (Å. Wieslander, L. Rilfors, L. B.-Å. Johansson, & G. Lindblom, unpublished experiments). This phase is formed by introducing surplus amounts of MGDG with pronounced wedge shape (i.e., containing cis unsaturated fatty acids) in the lamellar phase of DGDG. The observation further supports the self-assembly theory used.

It should be noted that the theory also nicely pertains to the effect of cholesterol on biological membranes. Monosugar diglycerides, forming a reversed hexagonal (H_{II}) phase

structure, are present in significant amounts in the membranes of all *Acholeplasma* species (Smith, 1979). The genus *Acholeplasma* is also characterized by having no requirement for cholesterol (Razin, 1978). Moreover, in resemblance to cell wall enclosed bacteria, *A. laidlawii* has a reduced capacity for cholesterol incorporation (Razin, 1975b). The presence of this molecule in the membrane diminishes the MGDG content (Table I). In light of the observed phase behavior of MGDG/DGDG mixtures (see above), it can be anticipated that large amounts of the wedge-shaped cholesterol molecule must induce a nonlamellar phase just as MGDG does. Hence, the simultaneous presence of large amounts of MGDG and cholesterol must be avoided. This finding is supported by the observation that cholesterol destabilizes the bilayer structure of dioleoylphosphatidylethanolamine as well as a mixture of soy phosphatidylethanolamine and egg yolk phosphatidylcholine (Cullis & De Kruijff, 1978a). Previously, the results obtained usually were discussed in terms of cholesterol as a moderator of "fluidity" or "microviscosity". This explanation has recently been questioned (G. Lindblom, L. B.-Å. Johansson, & G. Arvidson, unpublished experiments), since it was observed that the lipid lateral diffusion (a dynamic parameter) was practically unaffected by the presence of cholesterol in the bilayer. We propose that cholesterol, due to its molecular shape, exerts its greatest influence on bilayer structures by disturbing the packing of the lipid molecules.

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EFFECTS OF ANESTHETICS ON WATER PERMEABILITY AND LIPID METABOLISM
IN ACHOLEPLASMA LAIDLAWII MEMBRANES

by

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SUMMARY

The addition of tetracaine and diethylether to Acholeplasma laidlawii at concentrations commonly used for local anesthesia did not affect water permeability over the cell membrane, as measured by a pulsed magnetic field gradient NMR method. However, A. laidlawii changed its membrane lipid composition upon treatment with these anesthetics. Both tetracaine and diethylether addition resulted in a decrease in the molar ratio between the major membrane glucolipids, monoglucosyldiacylglycerol and diglucosyldiacylglycerol. The ratio between saturated and unsaturated acyl chains did not change. The results are in accordance with our proposal that A. laidlawii regulates its lipid composition in order to maintain optimal packing stability in the membrane (Wieslander, Å., Christiansson, A., Rilfors, L., and Lindblom, G. (1980) Biochemistry 19, 3650 - 3655). Introduction of anesthetics into the hydrophobic region of a bilayer is likely to affect the lipid packing. A membrane which contains lipids like monoglucosyldiacylglycerol, which forms a reversed hexagonal phase, will be destabilized unless the amounts of such lipids are reduced. The membrane concentration of anesthetics was estimated to one molecule per 12-15 lipid molecules. The fact that A. laidlawii regulates its lipid composition as a response to these concentrations, despite their negligible effect on water permeability, indicates a high sensitivity of this regulatory system.

INTRODUCTION

Every biological membrane contains a considerable number of different lipid molecules with different fatty acid compositions and polar head groups. The basis for this heterogeneity is only recently beginning to be understood. It is well known that the acyl chain composition of the lipids affects the physical properties of the bilayer [1] and the function of many membrane bound enzymes [2]. Recently the importance of the different polar head groups for the molecular packing in the bilayer [3,4,5] and for an optimal packing of proteins into the bilayer [6] has been proposed. Furthermore, lipids forming non-bilayer phases have been implicated in processes like membrane fusion and lipid flip-flop [7].

Acholeplasma laidlawii regulates its lipid composition as a response to changes in fatty acid composition, growth temperature and cholesterol [8,9,10,11]. The regulation involves not only changes in fatty acyl chain composition but furthermore an extensive regulation of polar head groups. We have recently proposed that this regulation is necessary for the cell to maintain optimal membrane stability and that the effects of environmental stimuli can be predicted on basis of their induced effects on the lipid packing in the bilayer [5]. This regulation keeps the balance between lipids forming lamellar and reversed hexagonal phase structures within certain limits, to maintain bilayer stability [4,5].

To further test the theory for regulation of lipid composition we have investigated whether the effects on lipid metabolism in A. laidlawii of membrane perturbing agents like anesthetics can be foreseen on basis of their disturbance of the bilayer. Anesthetics comprise a structurally very heterogeneous group of chemicals whose potencies to induce anesthesia in nerve membranes are roughly correlated with their lipid solubilities [12]. Reports have been presented on the ability of anesthetics to induce structural perturbations in both lipid bilayers and in proteins (for a review see [13]), though most theories hitherto seem to favour the action on lipids as the cause of anesthesia on nerve membranes. The introduction

of small molecules into the bilayer is likely to affect its permeability for ions [14] and water [15]. We report here on the use of NMR to measure water diffusion over cell membranes with a pulsed magnetic field gradient method. The effect of anesthetics on water diffusion has been compared to the physiological regulation of lipid composition occurring upon addition of anesthetics. All investigations have been performed at clinically relevant concentrations of anesthetics, hopefully to gain some insight into the mechanism of nerve cell anesthesia at a molecular level, using A. laidlawii as a model system.

MATERIALS AND METHODS

Organisms and growth conditions

A. laidlawii A, strain EF22, was grown at 37°C in a lipid-depleted tryptose/bovine serum albumin medium as described previously [11]. The medium was supplemented with 75 μ M of palmitic and 75 μ M of oleic acid, added as sterile ethanolic solutions. To label the membrane lipids 10 μ Ci/l [1-¹⁴C]oleic acid (56 mCi/mmol) and 30 μ Ci/l of [9,10-³H]palmitic acid (57,9 mCi/mmol) (The Radiochemical Centre) were added. Bacillus megaterium Ft R32, a facultatively thermophilic strain, was grown in a tryptone-starch broth [16] at 50°C. This strain was used in experiments where the effect of anesthetics on growth of a common bacterium and the wall-less Acholeplasma was compared. The organism were grown in side-arm flasks, containing different concentrations of anesthetics (see Table I). When volatile anesthetics were used, the flasks were closed with rubber stoppers, and a small volume of medium was contained in a big flask to assure accurate oxygen supply for the Bacillus cells. Growth was followed by measurement of absorbance at 540 nm.

To examine the effect of anesthetics on lipid metabolism, A. laidlawii was grown for 12 hours in a medium without anesthetics. The culture was divided in two halves and one half was supplemented with anesthetics, the other remained unsupplemented. The drugs used were diethylether (final concentration 0.05 M) and tetracaine (final concentrations 0.22mM). The drugs were added dissolved in fresh growth medium (c. 4 % of culture volume) and the unsupplemented culture received an equal volume of fresh

medium. To prevent the evaporation of diethylether, this experiment was performed in rubber-stoppered flasks. After supplementation, incubation was carried on and further growth was measured by absorbance at 540 nm. Samples for lipid analysis were taken at intervals. Cells were harvested by centrifugation at 32000xg for 10 minutes at 5°C. The cells were washed and membranes prepared as in [9].

Lipid analysis

Membranes were extracted with chloroform/methanol 2:1 (v/v) and the lipid extracts were purified as described earlier [9]. The individual polar lipid species were separated by thin-layer chromatography on silica gel H (Merck) plates (buffered with 1 % w/v $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) in chloroform/methanol/water 65:25:4 (v/v). The lipids were visualized by exposure to iodine vapour and were scraped off the plates into Pasteur pipettes stoppered with glass wool. The lipids were eluted with chloroform/methanol 2:1 (v/v) followed by methanol/formic acid 96:4 (v/v) into scintillation vials. After evaporation of the solvent, scintillation cocktail (Omnifluor-toluene, New England Nuclear Co.) containing 6 % (v/v) Biosolv (Beckman Instruments) was added and the samples were counted in a Nuclear Chicago (Mark II) liquid scintillation counter set for double label counting. Conversions from counts per minutes (CPM) to disintegrations per minute (DPM) were obtained by the external standard ratio method, using calibration curves produced by quenching ready-for-use internal standards (LKB-Wallac, Finland) in scintillation cocktail of the same composition. Quantification of acyl chain and polar lipid composition in A. laidlawii by determination of radioactivity in the fatty acyl chains has been shown to agree well with GLC measurements under the conditions employed [8], since virtually no endogenous fatty acid synthesis is present [11].

Preparation of NMR samples

A. laidlawii was grown for 18 hours at 37°C as described above and harvested by centrifugation (32000xg, 10 min.). The cells were washed in buffer (50 mM Tris, 130 mM NaCl, 2 mM MgCl_2 , 5 mM CaCl_2 , 1.2 mM Na_2HPO_4 , 1 mM KCl, 0.1 mM sucrose, 0.1 mM glycerol, pH 7.4). This

buffer permits no cell metabolism and has a stabilizing effect on the fragile A. laidlawii membrane [17]. After centrifugation the cells were resuspended in the same buffer with and without 2.23 mM tetracaine or 500 mM diethylether. The cell to drug ratio was the same as in the lipid metabolism study; see above. The suspensions were stirred very slowly for 30 minutes at room temperature and then centrifuged at 32000xg for 10 minutes. The obtained cell pellets were quantitatively transferred to graded NMR tubes, sealed with plastic stoppers and analyzed by NMR.

Determination of intra- and extracellular water

Cell pellets were prepared exactly as described above with the modification that the suspension buffer contained 0.16 $\mu\text{Ci/ml}$ [$\text{U-}^{14}\text{C}$] sucrose (610 mCi/mmol) and 0.4 $\mu\text{Ci/ml}$ [$2\text{-}^3\text{H}$]glycerol (500 mCi/mmol) (The Radiochemical Centre). After the last centrifugation, ^{14}C - and ^3H -activities were determined in the cell pellet and the supernatant, cf. [18]. The amounts of labelled sucrose adsorbed to the cell surface was determined by adsorbing the cells on 0.22 μm Millipore filters and washing them with the appropriate buffer. The fraction of intracellular water (versus extracellular water) was found to be approximately 20 % in the cell pellets intended for NMR analysis. This corresponds to roughly 1 μl of intracellular water per mg cell protein.

NMR diffusion measurements

All diffusion studies were done on a Bruker 322-s pulsed NMR-spectrometer operating at 61 MHz for protons. The spectrometer was equipped with a homebuilt pulsed magnetic field gradient unit. The water diffusion was measured in non-metabolizing cells with the pulsed magnetic field gradient method [19]. A $90^\circ\text{-}\tau\text{-}180^\circ$ pulse sequence was used. Two magnetic field gradients, spaced Δ ms, of equal strength δg , where δ is the duration and g the size of the gradient, were applied on each side of the 180° radiofrequency pulse. A spin-echo was observed after a time interval of 2τ from the start of the pulse sequence. Diffusion of water molecules during the time Δ (the diffusion time) attenuated the spin-echo amplitude according to the equation:

$$E_g/E_0 = \exp [-(\gamma \delta g)^2 \cdot (\Delta - \delta/3) \cdot D]$$

where E_g and E_0 are the spin-echo amplitudes with and without magnetic field gradients, γ is the magnetogyric ratio for protons and D is the self-diffusion coefficient for the diffusing species.

In the case of restricted diffusion i.e. when the translational diffusion of the water molecule is limited in space due to barriers of some kind, the observed diffusion coefficient reaches an asymptotic value for diffusion times several times larger than the time it takes a molecule to diffuse the distance between the barriers.

In a heterogenous system like the Acholeplasma cells there are two fractions of mobile water, intra- and extracellular water. The spin-echo attenuation in such a system can be described by a superposition of two exponentials [20,21]. Assuming that the exchange rates for water molecules across the cell membrane are large compared to the transverse relaxation rates for the intra- and extracellular water the echo attenuation will depend on diffusion only [20,21].

The NMR signal of the cell sample is comprised of a fast decaying component due to extracellular water, that has its transverse relaxation rate greatly enhanced by the magnetic field gradient and a slow decaying component due to intracellular water (less enhanced relaxation rate) with a decay rate partly determined by the mean residence time τ_r of water molecules inside the cells. If the time it takes for a water molecule to transverse the cell interior is small compared to the mean residence time of a water molecule within the cell, τ_r is determined by the water diffusional permeability of the cell membranes. ($\tau_r \geq \frac{r^2}{2D}$, r = cell radius, D = self-diffusion coefficient for water molecules inside the cell) [22].

I. Variation of the magnetic field gradient

The magnitude of the magnetic field gradient was varied in discrete steps from 0.4 to 4 T/m while Δ was kept constant in each series. The following values were chosen for Δ : 13, 20, 26, 33, 39 ms and τ was 10, 14, 20, 25, 30 ms respectively. δ was 1.5 ms in all measurements. The spin-echo amplitude was measured from an oscilloscope and $\ln(E_g/E_0)$ was plotted versus g^2 . These measurements were carried out at the following temperatures: 5^o, 10.5^o, 16.5^o, 25^o, 37.7^oC. The effect of tetracaine on the water permeability was studied. Restricted diffusion was also tested by investigating lysed cells (Fig. 1). A reference of water free glycerol was used to calibrate the magnetic field gradient unit. The same settings on the spectrometer were used for the cell and the reference samples.

In a NMR experiment when magnetic field gradient pulses of sufficient large strength are applied the fast decaying component of the signal is much attenuated and the spin-echo amplitude reaches a limit due to the restricted motion of the intracellular water. For large $\delta \cdot g$ the signal amplitude can be described by the following expression [22].

$$E_g/E_0 = p_b \exp [-(2\tau/\tau_r) - (\gamma \cdot g \cdot \delta \cdot r)^2/5]$$

where p_b is the intracellular water fraction. $\ln(E_g/E_0)$ is plotted vs. g^2 and its intercept at $g = 0$ is determined. This intercept is a function of τ and thus τ_r can be determined.

II. Variation of the diffusion time

In this kind of experiment g was kept at a constant value while Δ , the diffusion time, was varied in discrete steps from 10 to 60 ms. $\ln(E_g/E_0)$ was plotted vs. Δ and δ was 1.5 ms.

Diffusion measurements on water (with small amount of Cu^{++} added) were done in order to calibrate the magnitude of the magnetic field gradient unit. Self-diffusion coefficients for water at different temperatures were obtained from [23].

From these diffusion-studies we can determine a mean lifetime for water molecules inside the cells according to the method described by Andrasko

$$\tau_r = \frac{D_a - D(1 + p_b/p_a)}{(\gamma \cdot \delta \cdot g)^2 D(D_a - D)}$$

where D_a is the self-diffusion coefficient for water at 25°C ($D_a = 2.4 \cdot 10^{-9} \text{ m}^2 \text{ s}^{-1}$) p_a and p_b are volume fractions of extra- and intracellular water respectively. D is the observed diffusion coefficient obtained from the NMR measurements.

RESULTS

Growth in the presence of anesthetics

In order to choose some suitable membrane-active drugs we examined the growth response of A. laidlawii in the presence of some anesthetics at different concentrations. Since these agents are lipophilic it might be expected that a change in growth would reflect a perturbation in the membrane cf. [24]. Fig. 2 illustrates the results of a typical experiment with diethylether. With increased concentrations of diethylether in the growth medium A. laidlawii changed its growth rate from slightly stimulated at 0.05 M diethylether to zero at 0.5 M, where the cells lysed. It should be noted that 0.05 M diethylether is the nerve blocking concentration for frog sciatic nerves [12]. Like all mycoplasmas, but in contrast to other bacteria, A. laidlawii lacks a cell wall [25]. Since the presence of a cell wall connected to the cytoplasmic membrane might stabilize the membrane structure, a comparison of the growth of A. laidlawii and a common bacterium in the presence of membrane active drugs was performed. Table I illustrates the differences in sensitivity of A. laidlawii and B. megaterium Ft R32 to diethylether, ethanol (general anesthetics), tetracaine and procaine (local anesthetics). It can be noted that both organisms were affected by diethylether, ethanol and tetracaine in the range of concentrations used for local anesthesia. However, the concentrations required to inhibit B. megaterium were always

larger than those for A. laidlawii. Procaine inhibited growth of A. laidlawii below clinical concentrations, whereas B. megaterium remained growing until a hundredfold this concentration was applied. Detailed experiments were made with one general anesthetic, diethylether and with one local anesthetic, tetracaine.

Water diffusion

A comparison between the curves obtained in Fig. 1 strongly indicates restricted diffusion of water in intact cells. Almost free water diffusion occurred in the sample containing lysed cells. The water diffusion coefficients for this sample was found to be $4.1 \cdot 10^{-10}$ and $2.2 \cdot 10^{-10} \text{ m}^2/\text{s}$ at 28°C and 18°C , respectively. (cf. free water diffusion is $2.4 \cdot 10^{-9} \text{ m}^2/\text{s}$ at 25°C [23].) The mean residence times τ_r for water molecules in intact cells obtained at different temperatures are summarized in Table II. As can be inferred from this table the permeability increases with increasing temperature. From the data in Table II an apparent activation energy of 36 kJ/mol was calculated. The permeability $p_d = r/3\tau_r$ may be calculated using the average value of the cell diameter equal to $0.6 \mu\text{m}$. Our data are comparable to permeability values obtained for other systems [26,27]. The permeability may vary considerably depending on the lipid composition of a membrane [15].

The effect of tetracaine on the water permeability was also investigated. It was found that using the same batch of cells the mean residence time for water in cells supplemented with tetracaine ($\tau_r = 13^{+4} \text{ ms}$) did not differ significantly from those without the drug ($\tau_r = 12^{+4} \text{ ms}$). Similar results were obtained by using the method by Andrasko [21] on cells supplemented with diethylether.

Effects of anesthetics on lipid metabolism

To investigate the influence of anesthetics on water permeability cells were exposed to the drugs in a buffer. Due to the lack of nutrients in the buffer, the cells should not be able to compensate metabolically for the introduction of anesthetics into the membrane. Any disturbance created would be measurable without the masking influence of lipid

compensating mechanisms. To examine the extent of metabolic compensation we employed a shift technique, adding an anesthetic to one half of an actively growing culture and leaving the other half unsupplemented.

After addition of anesthetics growth continued uninterrupted in both the diethylether culture and the tetracaine culture. There was a slight decrease in the growth rate of the diethylether culture compared to the control, whereas 0.22 mM tetracaine did not affect growth at all. Fig. 3 demonstrates the responses in lipid metabolism upon addition of anesthetics. During growth of the control cells there was a gradual decrease in the monoglucosyldiacylglycerol/diglucosyldiacylglycerol ratio due to an increased incorporation of oleic acid into the lipids, cf. [8,9]. Immediately after the shift the glucolipid ratio decreased even further, (Fig. 3A,B). This decrease was small compared to the magnitude of changes occurring upon temperature shift-down [9], but reproducible. Upon further growth the glucolipid ratio of the diethylether culture approached that of the control (Fig. 3A), whereas that of the tetracaine culture was larger than the control after 12 hours of growth (Fig. 3B). The results obtained for the diethylether culture can probably be attributed to the difficulties to maintain the proper ether concentration after repeated samplings. Virtually no change occurred in the relative amount of ionic lipids of the diethylether culture when compared with the control (Fig. 3C). On the contrary, after addition of the positively charged tetracaine there was an increase of ionic lipids (negatively charged) (Fig. 3D). Analysis of total acyl chain composition revealed practically no differences (less than one per cent difference) between the anesthetic supplemented cultures and the controls.

DISCUSSION

Previously we have proposed that variation in lipid composition is an important factor in maintaining optimal membrane stability in A. laidlawii [5]. The individual lipids in the bilayer can be visualized as building bricks with different geometries. Important factors determining the shape of the bricks are the volume of the hydrophobic part of the

molecules, the hydrocarbon chain lengths and the optimal surface areas occupied by the polar head groups. These bricks will determine the shape of the aggregates formed [3]. Lipids with different molecular shapes may be accommodated in a bilayer as long as they can pack together to form a stable lamellar structure [4,5]. The major lipids in A. laidlawii are monoglucosyldiacylglycerol and diglucosyldiacylglycerol. Monoglucosyldiacylglycerol is characterized by a small polar head group compared with the hydrophobic part, resulting in a reversed hexagonal (H_{II}) phase of the pure lipid in water, whereas diglucosyldiacylglycerol with a larger head group forms a lamellar phase [4,5,28]. One of the main lipid regulation mechanisms in A. laidlawii concerns the molar ratio monoglucosyldiacylglycerol/diglucosyldiacylglycerol. It has been found that in vitro mixtures of monoglucosyldiacylglycerol/diglucosyldiacylglycerol with ratios corresponding to those found in vivo form a lamellar phase, whereas larger ratios yield nonlamellar phases [29]. Another mechanism concerns the amount of ionic lipids (phosphatidylglycerol, glycerophosphorylmono- and diglucosyldiacylglycerol) in the membrane. Introduction of charges in a polar head group will change the optimal surface area and thus the packing properties of a lipid.

In the present investigation changes in acyl chain composition caused by addition of anesthetics were negligible and the results can therefore not be attributed to packing disturbances introduced by changes in acyl chain saturation. The lipophilic anesthetics, as shown for tetracaine [30,31], penetrate into the hydrocarbon region of the bilayer, and cause packing disturbances. This should lead to an increase in the hydrophobic bulkiness and a bilayer containing lipids with H_{II} -tendencies, e.g. monoglucosyldiacylglycerol, should be destabilized, cf. [32]. Cholesterol, which also increases the hydrophobic volume of bilayers, was shown to induce H_{II} phase formation in in vitro mixtures of mono- and diglucosyldiacylglycerol [33]. A. laidlawii can therefore be expected to decrease the glucolipid ratio. Likewise, introduction of small molecules into the bilayer will dilute the charged lipids present, leading to a decrease in membrane surface charge [5,34]. We have shown previously that A. laidlawii regulates the amount of ionic lipids upon similar changes.

Fig. 3A,B illustrates the regulation of the monoglucosyldiacylglycerol/diglucosyldiacylglycerol ratio upon addition of anesthetics. In both experiments the molar ratio decreased as predicted. No regulation of the amount of ionic lipids occurred in the diethylether culture (Fig. 3C). This can be explained by the fact that the diethylether concentration in the membrane was very low. Using membrane/buffer partition coefficients from Seeman and Leo et al. [12,35] a rough estimate gives one anesthetic molecule per 12 - 15 lipids for both diethylether and tetracaine. This is equal to one molecule per approximately $800 \text{ \AA}^2$ and the changes in surface charge introduced by the diethylether molecules may be small enough not to require compensation. Contrary to diethylether, the tetracaine shift resulted in a significant increase in the amount of ionic lipids (Fig. 3D). This increase seems to compensate for the amount of tetracaine molecules present in the membrane. Since the tertiary amine group of tetracaine is positively charged at the pH of the growth medium, its presence in the Acholeplasma membrane will lead to a charge neutralization when it interacts with the negatively charged ionic lipids. This will effectively reduce the optimal surface area per lipid molecule and may lead to a bilayer destabilization. Thus the combined effects of tetracaine on the surface area and the hydrophobic volume may not be adequately compensated for merely by a decrease in the glucolipid ratio, but it also regulated by an increase in ionic lipids. A structurally related anesthetic, dibucaine, has been shown to induce formation of a H_{II} phase when interacting with diphosphatidylglycerol [36]. It can thus be concluded that the observed lipid metabolic regulations pertain nicely to the proposed theory. However no changes in water permeability was detected in non-metabolizing cells upon addition of anesthetics. This indicates that the disturbances introduced at clinical concentrations of anesthetics were too small if any to be measured. Similar conclusions have been reached for lipid membrane model systems using other physical methods, e.g. X-ray and neutron diffraction [38], NMR [39] and ESR [40]. However, although not always measurable, the introduction of anesthetics into a bilayer should lead to a concentration-dependent increase in the disorder or entropy of the system, as shown theoretically by Hill [41]. Since the cytoplasmic membrane is the only barrier to the outer milieu in A. laidlawii, it can be expected that a very sensitive lipid

regulating systems should be at work to ensure optimal packing stability and functional intactness of the membrane. It is of interest to note that B. megaterium having a stabilizing cell wall outside the cytoplasmic membrane, was less sensitive to anesthetics than A. laidlawii (Table I).

The effects on lipid regulation observed in A. laidlawii upon addition of anesthetics may be relevant to the explanation of nerve cell anesthesia. The balance between lamellar and non-lamellar phases in mixtures of A. laidlawii glucolipids is very sensitive to changes in the hydrophobic region of the lipids [29,33], and is regulated upon addition of anesthetics, to maintain optimal stability of the lamellar phase. Very subtle changes can affect the phase structure of a lipid and thus its packing in a membrane, cf. [42]. In a biological membrane the geometry of lipids are important for the correct embedding and probably function of membrane proteins [6]. In contrast to A. laidlawii nerve cells normally exist in a very constant environment and are not designed to rapidly change their lipid composition upon contact with e.g. anesthetics. The induced changes in the lamellar phase stability (or instability) might therefore be of crucial importance for the function of gating mechanisms regulating the transverse ion fluxes of the nerve impulse.

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TABLE I

DIFFERENTIAL EFFECT OF MEMBRANE-ACTIVE DRUGS ON GROWTH OF ACHOLEPLASMA LAIDLAWII AND BACILLUS MEGATERIUM Ftr 32.

The drugs were added to the growth media (see materials and methods for composition) before inoculation. Growth temperatures were 37°C and 50°C respectively, and growth was measured by absorbance at 540 nm. The growth rates are expressed in % of control cultures without drugs.

(A) Diethylether (0.05M) ^{a,b}		(B) Ethanol (0.5M) ^b	
Conc. (M)	Relative growth rate (%) <u>A. laidlawii</u> <u>B. megaterium</u>	Conc. (M)	Relative growth rate (%) <u>A. laidlawii</u> <u>B. megaterium</u>
0.05	108 100	0.25	- 93
0.075	44 -	0.50	0 68
0.10	31 100	0.63	0 48
0.25	- 92	0.75	0 0
0.50	0 0		

TABLE I cont.

(C) Tetracaine (0.1-0.01mM) ^{c,d}			(D) Procaine (4.6mM) ^b		
Conc. (mM)	Relative growth rate (%)		Conc. (mM)	Relative growth rate (%)	
	<u>A. laidlawii</u>	<u>B. megaterium</u>		<u>A. laidlawii</u>	<u>B. megaterium</u>
0.11	97	100	1.9	62	100
0.22	65	-	3.8	5	100
0.25	-	85	19	0	100
0.50	-	62	46	0	100
0.66	2	-	115	-	84
0.75	-	0	230	-	34
			460	-	0

a) Figures in parenthesis denote nerve-blocking concentrations

b) Data taken from Seeman [12], obtained with frog sciatic nerve

c) Data taken from Seeman [12], obtained with squid giant axon (0.1mM)

d) Data from Skou [37], obtained with frog sciatic nerve

TABLE II

THE MEAN RESIDENCE TIME FOR WATER MOLECULES INSIDE ACHOLEPLASMA LAIDLAWII
CELLS AT DIFFERENT TEMPERATURES

t (°C)	5.0	10.5	16.5	25.0	37.7
τ_r (ms)	72	47	33	19	12

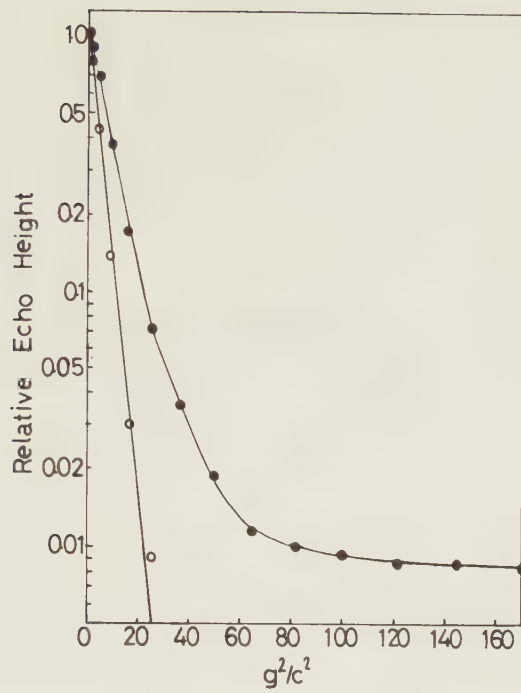


Fig. 1 Relative echo height vs. g^2/c^2 ($c=0.37$ T/m) for lysed cells (○) and for intact cells (●) at 28°C . Δ was kept constant at 26 ms, δ was 1.5 ms and τ was 20 ms.

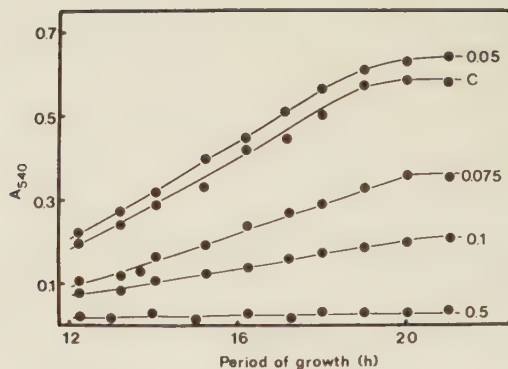


Fig. 2 Effect of diethylether on growth of *Acholeplasma laidlawii*. Cells were grown at 37°C in a lipid-depleted tryptose-bovine serum albumin medium supplemented with 75 μ M of palmitic and 75 μ M of oleic acid. Different concentrations of diethylether (conc. in M) were added before inoculation. C = control culture without addition. Growth was estimated by absorbance measurements at 540 nm.

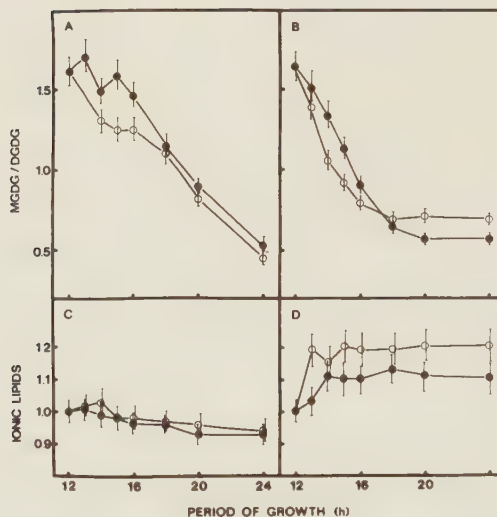


Fig. 3 Regulation of lipid composition in *A. laidlawii* upon addition of anesthetics. Cells were grown at 37°C for 12 hours. At this time the culture was divided in two parts, one of which was supplemented with anesthetics (○) while the other remained unsupplemented (●). A and B: molar ratio monoglucosyldiacylglycerol/diglucosyldiacylglycerol. C and D: relative amount of ionic lipids. The percentage of ionic lipids in the membranes at 12 hours was assigned the value of one. A and C: 0.05 M diethylether. B and D: 0.22 mM tetracaine. Lipids amounts were determined by liquid scintillation counting. Error bars denote 95 % confidence limits determined by propagation of error.

PROTEIN COMPOSITION AND EXTRACTABILITY OF LIPID-MODIFIED MEMBRANES
FROM *ACHOLEPLASMA LAIDLAWII*[†]

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RUNNING TITLE: SOLUBILITY OF LIPID-MODIFIED MEMBRANES

Abbreviations

CFU, colony-forming units

CIE, crossed immunoelectrophoresis

DGDG, diglucosyldiglyceride

H_{II}, reversed hexagonal phase

MGDG, monoglucosyldiglyceride

NaDodSO₄, sodium dodecyl sulfate

NaDodSO₄ PAGE, sodium dodecyl sulfate polyacrylamide gel
electrophoresis

NaDOC, sodium deoxycholate

PE, phosphatidylethanolamine

PG, phosphatidylglycerol

Tw 20, Tween 20

TX-100, Triton X-100

Abstract

Membranes from *Acholeplasma laidlawii* have been extracted with neutral detergents, which solubilize the proteins and lipids *selectively*, or with sodium deoxycholate, which gives an almost total solubilization. The amounts of individual proteins present in the detergent extracts of membranes with induced variations in lipid compositions were determined by crossed immunoelectrophoresis. Extraction with the neutral detergent Tween 20 showed that ionic lipids and the glucolipid diglucosyldiglyceride were enriched in the Tween extracts, whereas the glucolipid monoglucosyldiglyceride (which cannot easily be accommodated in micelles for geometrical reasons) was enriched in the membrane residue. The amount of monoglucosyldiglyceride in the Tween 20 extracts increased when the content of this lipid was increased in the membrane and protein D_{12} was also more easily extracted from membranes rich in monoglucosyldiglyceride. This was not correlated with an increase in the *total* amounts of D_{12} in the membrane (as analyzed by crossed immunoelectrophoresis after sodium deoxycholate solubilization), indicating that monoglucosyldiglyceride is involved in the anchoring of protein D_{12} in the membrane. The *total* amount of the flavoprotein T_{4a} in the membrane was found to increase upon enhanced amounts of ionic membrane lipids. Furthermore, protein T_{4a} was found to be increasingly *extractable* upon an increase in the amounts of unsaturated fatty acyl chains in the lipids. Several other proteins also displayed lipid-dependent extractabilities. These results support the hypothesis that for membrane proteins the extractability with neutral detergents and thus, interactions with lipids, are partly dependent upon the molecular shapes of the membrane lipid molecules. Thus, by use of these selective extraction procedures, information about protein-lipid interactions in the membrane was gained.

Membrane proteins are dependent upon an interaction with the lipid matrix, for optimal function. However, few membrane proteins have been shown to have requirement for specific lipids (Gennis & Jonas, 1977; Sandermann, Jr., 1978). Existence of different lipids in a membrane is probably necessary for a correct embedding of different proteins in the bilayer (Israelachvili, 1977), since the conformation of the hydrophobic parts of integral membrane proteins probably differ considerably between one protein and another. The shapes of these hydrophobic parts cause geometric packing constraints in the bilayer and may impose structural couplings between proteins and their lipid surroundings (Israelachvili, 1977). It is likely that no unperturbed lipid bilayer regions exist in biological membranes since they in general have a high protein/lipid ratio (Israelachvili *et al.*, 1980). Geometrical factors are also considered to be very important for the assembly of amphiphilic molecules into membrane bilayers and other lipid structures (Israelachvili *et al.*, 1976). They further set the limits to lipid variation in a membrane corresponding to a *stable* lamellar (bilayer) phase. The extensive variation in polar lipid composition that occurs in *Acholeplasma laidlawii* upon internal and environmental stimuli is a mechanism for securing a stable bilayer (Wieslander *et al.*, 1980; 1981). The dominant changes occurred in the ratio of the main polar lipids monoglucosyldiglyceride (MGDG)¹ and diglucosyldiglyceride (DGDG)¹, which in pure state form a reversed hexagonal (H_{II})¹ and a lamellar phase, respectively, together with water (Wieslander *et al.*, 1978).

By extensive induced variations in the lipid matrix the structural couplings between proteins and lipid in membranes are likely to be changed (*cf.* Borochoy & Shinitzky, 1976). Several investigations have indicated that variations in membrane protein composition can occur in *A. laidlawii* upon imposed changes in the acyl chain content of the membrane lipids (Morowitz & Terry, 1969; Pisetsky & Terry, 1972; Amar *et al.*, 1979; Silvius *et al.*, 1980). However, no individual proteins were identified.

The specificity and the quantitative nature of antigen-antibody reactions often make immunological methods superior to others in membrane studies (Bjerrum, 1977; Owen, 1981). Several individual integral membrane proteins of *A. laidlawii* have been purified, localized and partially characterized by selective detergent solubilization and electroimmunochemical methods (Johansson & Hjertén, 1974; Johansson *et al.*, 1979). The identity of the main precipitates obtained by crossed immunoelectrophoresis (CIE)¹ of the solubilized *A. laidlawii* membrane proteins have also been established (Johansson & Wróblewski, 1978).

By extracting *A. laidlawii* membranes with a nonionic surfactant, e.g. Tween 20 (Tw 20)¹, a selective solubilization of integral proteins occurs (cf. above). The mechanism for solubilization with nonionic detergents can be understood on a geometrical basis. When inserted into a bilayer the detergent molecules locally increase the curvature of the membrane by way of their pronounced wedge shape (V, with polar head at top) and causes a (local) lamellar to micellar transition (Helenius & Simons, 1975).

In this work the influence of membrane lipid composition on total amounts and extractability of membrane proteins from *A. laidlawii* B(ju) have been studied by sensitive electroimmunochemical methods. It was found that both the total amounts of certain individual proteins in the membrane, as well as their detergent extractability, are partly dependent upon the molecular shapes of the membrane lipid molecules. The most pronounced effect was observed for a membrane bound flavoprotein.

Experimental Procedures

Organism and growth conditions. *Acholeplasma laidlawii*, strain B(ju), was grown statically at 30°C in thoroughly lipid-depleted bovine serum-albumin/tryptose media (Christiansson & Wieslander, 1980), supplemented with palmitic and oleic acids in the following

proportions ($\mu\text{M}/\mu\text{M}$): 0/150, 30/120, 75/75 and 120/30. A mixture of oleic acid, linoleic acid and cholesterol was obtained by supplementing the medium (containing no bovine serum albumin) with 1% (v/v) of PPLO Serum Fraction (Difco Laboratories). The cells were well adapted to the different supplements by at least 10 consecutive passages. Membrane lipids were labelled by adding 10 $\mu\text{Ci}/\text{l}$ of $[^{14}\text{C}]$ -oleic acid (56 Ci/mol) to *all* media. The PPLO serum fraction medium was also supplemented with 10 $\mu\text{Ci}/\text{l}$ of $[^{14}\text{C}]$ -linoleic acid (51 Ci/mol) and 40 $\mu\text{Ci}/\text{l}$ of $[^3\text{H}]$ -cholesterol (43 Ci/mmol). The palmitic acid containing media were supplemented with 30 $\mu\text{Ci}/\text{l}$ of $[^3\text{H}]$ -palmitic acid (57.9 Ci/mol).

Growth was followed by light microscopy, measurement of pH and the optical density at 400 nm and by determination of colony-forming units (CFU)¹.

Preparation of Membranes. Membranes were prepared by osmotic lysis (Johansson *et al.*, 1975) and stored in diluted (1:20) β -buffer (Razin *et al.*, 1965) at -70°C until used.

Materials. Agarose A was obtained from Pharmacia Fine Chemicals. Sodium dodecylsulfate (NaDodSO_4)¹ was purchased from Kebo AB, Stockholm, Sweden. Sodium deoxycholate (NaDOC)¹ and Triton X-100 (TX-100)¹ from Sigma and Tween 20 ($\text{Tw } 20$)¹ from Atlas Chemie GmbH, Essen, Germany.

Solubilization of Membranes with Different Detergents. The following four detergent solutions were used: NaDodSO_4 (0.2 M), TX-100 (5% w/v), and Tw 20 (5% w/v) in 0.1 M Tris-HCl buffer (pH 8.0) and NaDOC (0.12 M) in 0.1 M glycine-NaOH buffer (pH 9.1). A volume of 50 μl of a membrane suspension containing 40 mg/ml of protein and lipid was mixed with 50 μl of the detergent solution. With respect to Tw 20, the final concentration in the membrane-detergent mixture, *i.e.* 2.5% (w/v), corresponds to approximately 1.2 mol of Tw 20 per mol of membrane lipids, *c.f.*

Simons *et al.* (1973). The mixtures were left for 1 h at room temperature and insoluble material was then sedimented by centrifugation at $130\,000 \times g$ for 10 min in a Beckman[®] Airfuge^{T.M.}

The supernatants, which will be referred to as e.g. the NaDOC supernatant, were stored at -70°C until used. Total protein content was estimated according to Hartree (1972) and by measuring the absorbance at 280 nm. Tw 20 does not contribute significantly to the absorbance at 280 nm.

Polyacrylamide gel electrophoresis. Protein composition of membranes and extracts were analyzed by NaDodSO_4 polyacrylamide gel electrophoresis (PAGE)¹ in a discontinuous buffer system as described (Wieslander *et al.*, 1979). Resolution was enhanced by preparing a 7-22.5% (w/v) linear gradient of acrylamide with 1% crosslink.

Production of antisera. Polyspecific antisera directed against the membrane proteins were raised in rabbits by inoculation with 0.4 mg of membranes per immunization (Harboe & Ingild, 1973). Monospecific antisera were produced against the proteins T_2 , T_{4a} , and D_{12} (Johansson *et al.*, 1979; Johansson & Wróblewski, 1978) by immunizing rabbits with excised immunoprecipitates (Watson & Wildly, 1969) obtained by preparative line immunoelectrophoresis (Krøll, 1973) of the purified proteins.

Crossed immunoelectrophoresis. The amounts of individual membrane proteins in the detergent supernatants were determined by crossed immunoelectrophoresis (CIE)¹ (Laurell, 1965) in the presence of detergent (Johansson & Hjertén, 1974) with the modifications given in Table I. Peak areas subtended by individual immunoprecipitates were estimated by weighing the excised drawings obtained after magnification (100 X) in a standard photographic enlarger. The identity of some immunoprecipitates were established

by CIE with intermediate gels containing monospecific antisera (Axelsen, 1973). In the CIE-NaDOC system only monospecific antisera were used. The concentration of the antisera was in general $10 \mu\text{l}/\text{cm}^2$ and the thickness of the antibody-containing gel was 1.2 mm. The titre of the antiserum against T_{4a} was lower than the titres of the other monospecific antisera. Thus, the concentration of this antiserum in the CIE experiments was $20 \mu\text{l}/\text{cm}^2$.

Lipid analysis. Lipids were extracted from membranes and residues with a modified Bligh & Dyer method according to Kates (1972). Excess of unlabelled carrier lipids (from *A. laidlawii*) were added to the extraction mixtures. Separation by thin-layer chromatography, identification, purification, and quantification by gas-liquid chromatography and liquid scintillation counting were performed as described (Christiansson & Wieslander, 1978; 1980) with the modification that 6% (v/v) Biosolv[®] (Beckman) was included in the liquid scintillation cocktail.

Results

Solubilization conditions

The impact of several experimental factors were established for membranes from cells grown with $75 \mu\text{M}$ each of palmitic and oleic acids. As judged from CIE, all detectable proteins were more efficiently solubilized with Tw 20 than with TX-100. This difference was observed independently of the presence of Tw 20 or TX-100 in the gels. Extraction of membranes with Tw 20 at different pH (between 8.0 and 9.1) did not affect the immunoprecipitation pattern in the Tw 20 system (see Table I) significantly. No drastic changes in relative concentrations of

the proteins T₂, T_{4a} and D₁₂ were observed by CIE-NaDOC of NaDOC supernatants of membranes from cells harvested at different phases of the growth cycle.

Solubilization of membranes with different lipid compositions. By changing the fatty acid composition of the growth medium extensive variation of membrane polar lipid composition can be introduced. These variations are much more pronounced than variations occurring within a growth cycle (Wieslander & Rilfors, 1977; Wieslander *et al.*, 1980). Table II shows the lipid composition in membranes from *A. laidlawii* B(ju) grown to the late log phase with different amounts of saturated and unsaturated fatty acids. Great differences in lipid composition were revealed. When small amounts of unsaturated fatty acids (*i.e.* oleic acid) were incorporated, MGDG synthesis increased and the amounts of DGDG and ionic lipids decreased (Table II). This B strain thus possesses the same regulating mechanisms for polar lipid composition as strain A of *A. laidlawii* (Wieslander *et al.*, 1980). Although significant variations occurred in the ratio lipid/protein (Table II), NaDodSO₄ PAGE patterns of membrane protein were almost identical (Figure 1). These membranes were also extracted with Tw 20 and NaDOC (see Experimental procedure). Table III shows that 50-90% of the membrane was solubilized with Tw 20 and 70-100% with NaDOC, as judged from absorbance measurements at 280 nm. The membrane preparations, which were most soluble in NaDOC (corresponding to samples III and IV) were also solubilized to a great extent with Tw 20. Protein composition in NaDOC supernatants of membranes I-V was almost indistinguishable from that in the corresponding membranes (Figure 1) as analyzed by NaDodSO₄ PAGE (data not shown).

NaDodSO₄ PAGE of proteins in the Tw 20 insoluble residues obtained after Tw 20 extraction revealed a more individual pattern (data not shown) than that shown for intact membranes, *i.e.* Figure 1, or NaDOC supernatants. The differences in protein composition after Tw 20 extraction are more easy to quantify by CIE analysis of Tw 20 supernatants, see Figure 2. Note that the relative proportions of several proteins vary significantly, much more than what can be seen in Figure 1. Most likely, this is caused by differences in affinity of the proteins for the Tw 20/lipid mixed micelles or the membrane residues, respectively. It should be noted that the relative electrophoretic rates of migration in the first dimension on the CIE plates were the same for proteins T_{4a}, T₃, T₂, t_{1a} (= D₁₂) and t_{1b} (Figure 2), which was not always the case when Tw 20 supernatants of membranes from different strains of *A. laidlawii* were analyzed by CIE (Steinick *et al.*, 1980).

Selective solubilization was also observed for membrane lipids. Table IV shows the lipid composition in membrane residues after Tw 20 extraction. A comparison with the compositions in intact membranes (Table II) reveals that unsaturated lipids are slightly enriched in the Tw 20 supernatant, which also applied for ionic lipids, DGDG and cholesterol. However, MGDG was highly enriched in the Tw 20 insoluble membrane residue (Table IV). The amount of total proteins or lipids transferred into Tw 20 micelles seems to depend on the lipid/protein ratio in intact membranes, *i.e.* a low ratio yields a protein-enriched Tw 20 supernatant (cf. Table II & IV).

Concentration of individual proteins in membranes and detergent extracts. The solubility of both membrane proteins and lipids by detergents was affected by membrane lipid composition (see above). Variation of protein composition in Tw 20 supernatants can be

caused by a variation in amounts of individual proteins in the membrane and/or by a difference in extractability of a certain protein, caused by variations in lipid organization around that protein. Therefore, the content of some of the major membrane proteins was determined by CIE of Tw 20 supernatants and NaDOC supernatants. Figure 3 shows the relative amounts of these proteins in Tw 20 supernatants expressed as percentage of the amount in sample No. I. The sample numbers refer to those used in Table II. All proteins investigated were most easily extracted from sample No. I, *i.e.* membranes from cells grown in the presence of PPLO serum fraction (Figure 3). The content of the proteins T₂, T_{4a}, and D₁₂ in NaDOC supernatants was determined by CIE against the corresponding monospecific anti-serum. Figure 2 F illustrates one representative experiment. Figure 4 shows the relative amounts of these proteins expressed as percentage of the amount in the sample containing the highest concentration. Since protein composition in NaDOC extracts was very similar to that in membranes, Figure 4 illustrates total variations of these proteins in the membrane preparations with different lipid compositions. However, the amounts given in Figure 3 represents the solubility of the particular protein in Tw 20. Comparison with Table II reveals that the extractability (Figure 3) of protein T_{4a} is proportional to the amount of unsaturated acyl chains or amount of ionic lipids in the membranes. For protein D₁₂ large amounts of H_{II} lipids (MGDG and cholesterol) in the membranes correlates with large extractability (Figure 3 & Table II). The total amounts of protein T_{4a} in the membranes (Figure 4) correlates with the amounts of ionic lipids present in the membranes (Table II). This variation of T_{4a} in the membrane is representative for whole

cells since analysis of cytoplasmic fractions by CIE revealed no T_{4a} (data not shown).

The concentration of protein T_2 in the Tw 20 supernatant (Figure 3) was *not* proportional to its relative amount in the membrane (Figure 4), indicating that the extractability of this protein is also dependent upon its lipid surroundings.

Discussion

Solubilization of lipids. The rules governing the selective extraction of membrane protein with detergents are probably very complex. Protein-protein interactions may be important for this selectivity when mild detergents are used. To some extent, however, the selectivity can also be rationalized on the basis of the interactions between the proteins and the lipid matrix (Helenius & Simons, 1975). These interactions are limited by packing constraints that are dependent upon the molecular geometry of the surrounding lipid molecules, cf. Figure 1 and 2 in Israelachvili (1977). Furthermore, the action of neutral detergents can be explained by their pronounced wedge shape geometry.

By changing the polar lipid composition in the *A. laidlawii* membranes, the sizes of lipid domains and their interactions with membrane proteins are likely to vary. Many membrane proteins are selectively solubilized with Tw 20, cf. Figure 1 and 2, see also Johansson *et al.* (1979) and Steinick *et al.* (1980). This selectivity must depend on the distribution of a certain protein (and its lipid surroundings) between the lamellar (membrane) and micellar phase. MGDG (molecular shape Δ , with polar head at top) and neutral detergents have geometrical properties, which are complementary. However, the shape of MGDG is probably energetically very unfavourable in a Tw 20 micelle due to the

high curvature of the mixed aggregate. Therefore, MGDG is less likely to take part in the lamellar to micellar transition. Table IV reveals a great enrichment of MGDG in membrane residues after Tw 20 extraction. A high degree of unsaturation results in a more accentuated wedge shape of MGDG (see Wieslander *et al.*, 1980; 1981). The most unsaturated supplement (sample No. I) yields the highest proportional enrichment of MGDG in the Tw 20 residue (Table IV). Furthermore, this residue is depleted of cholesterol, probably as a consequence of the packing mismatch between MGDG and cholesterol, cf. Khan *et al.* (1981) and Israelachvili *et al.* (1980). Similar ethanolamine/phosphatidyl (PE)¹ observations have been made with other lipids, e.g. phosphatidyl (PE)¹ is enriched in the TX-100 insoluble residue of Semliki Forest Virus (Simons *et al.*, 1974).

The selective lipid enrichment observed here after extraction of *A. laidlawii* membranes with Tw 20 is much more pronounced than what has been observed after extracting erythrocyte membranes with similar amounts of TX-100 (*i.e.* molar ratio detergent/membrane lipid 1:1) (MacDonald, 1980). However, different neutral detergents (and lipids) differ considerably in physical and solubilizing properties (Egan *et al.*, 1976).

Solubilization of proteins. Protein D₁₂ is proportionally extracted by Tw 20 from the *A. laidlawii* membranes as a function of their H_{II} lipid content, but *not* more abundant in such membranes (cf. Table II and Figure 3 and 4). It should be pointed out that most of D₁₂ is enriched in the Tw 20 insoluble membrane residue, see Results and Johansson & Wróblewski (1978), which also holds for MGDG. However, more MGDG in the membranes yields more MGDG and D₁₂ in the Tw 20 supernatants (Table II and IV). This indicates that MGDG might be important for the anchoring of D₁₂ in the membrane.

It is known that ionic amphiphilic molecules like anionic polar lipids (e.g. PG) more easily form mixed micelles together with neutral detergents, than zwitterionic or nonionic polar lipids (Tanford, 1973; Israelachvili *et al.*, 1976). Table II and IV reveal an enrichment of ionic lipids in Tw 20 micelles. A comparison of Figure 4 and Table II also shows a positive correlation between the amounts of ionic lipids in the membranes and the amounts of protein T_{4a} . However, for T_{4a} the extractability diminishes faster with decreased amounts of unsaturated acyl chains in membrane lipids than does the actual amounts of T_{4a} and ionic lipids in the membranes (see Table II and IV and Figure 3 and 4). The lipids in the least unsaturated sample (*i.e.* V) are probably in the gel state during the extraction procedure (*cf.* Wieslander *et al.*, 1978). A high degree of molecular ordering thus seems to make T_{4a} less extractable.

It is assumed that the specificity of protein solubilization occurs during the primary micellarization in the membrane (Helenius & Simons, 1975). This is supported by the finding that no transfer of protein occurs from *A. laidlawii* membranes to various lipid aggregates (Kahane & Razin, 1977; Slutzky *et al.*, 1977; Razin *et al.*, 1980).

Variation of protein amounts in membranes. By increasing synthesis of ionic lipids, *A. laidlawii* can compensate for increased amounts of unsaturated acyl chains in the lipids, probably in order to maintain the surface charge density and the stability of the lamellar (bilayer) phase (Wieslander *et al.*, 1980). The variation of T_{4a} amounts in the membranes (Figure 4) probably does not involve any incorrect insertion of this integral protein (*cf.* Tyhach *et al.*, 1979), or a limited number of binding sites (*cf.* Kung & Henning, 1972)

since we could not detect T_{4a} (with CIE) in the cytoplasmic fractions of the cells. This major membrane flavoprotein T_{4a} (Johansson *et al.*, 1979) is probably a very important protein in the truncated electron transport chain of *A. laidlawii* (Razin, 1979). It has been shown that the passive permeability of *A. laidlawii* membranes and derived lipids increases dramatically with increased amounts of unsaturated fatty acyl chains in the lipids (summarized by Razin, 1975). Thus, the increase in T_{4a} amounts in unsaturated ("permeable") membranes might be a mechanism to maintain a certain membrane potential at conditions with a larger leakiness. Variation in membrane proteins and electron transport chain constituents upon changes in environmental conditions, *e.g.* reduction/oxidation potential and metabolic status, is very common in other bacteria, *cf.* Gel'man *et al.* (1975) and Mallick & Herrlich (1979). It is known that phage infection of *E. coli* causes a disturbance of membrane energetization (Braun & Oldmixon, 1979). Infection of *A. laidlawii* with mycoplasma virus group 2 (MVL 2) greatly enhances the amounts of protein T_{4a} found in a Tween 20 extract of membranes (Steinick *et al.*, 1980), supporting a regulation of T_{4a} amounts in the membrane.

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Table I: Detergent systems used in crossed immunoelectrophoresis

Detergent	Conc. of detergent in the gel		Buffer	Field strength 1st dimension ^c	Duration of the run 1st dimension ^c	Antiserum
	1st dimension	2nd dimension				
Tw 20 or TX-100	1% (w/v)	1% (w/v)	80 mM Tris-acetic acid (pH 8.6)	15 V/cm	50 min	Polyspecific ^d
NaDOC ^a	12 mM	0 mM	0.1 M glycine-NaOH ^b (pH 9.1)	10 V/cm	20 min	Monospecific

^a cf. Wróblewski *et al.*, 1977.

^b Gives sharper immunoprecipitates in the presence of NaDOC, than the Tris buffer.

^c The field strength and the duration of the run for the *second* dimensional electrophoresis were 2 V/cm and 15-20 h, respectively.

^d In some experiments intermediate gels containing a monospecific antiserum were used.

Table II: Lipid composition in membranes from *A. laidlawii* B(ju) grown with different amounts of saturated and unsaturated fatty acids (24 h, 30°C)

Sample no.	I	II	III	IV	V
Supplementation of palmitic and oleic acid ($\mu\text{M}/\mu\text{M}$) to the growth medium	PPLO ser. fract. ^a	0/150	30/120	75/75	120/30
% oleic acid in lipids (mol/mol)	60 (+25% 18:2c) ^b	95	84	57	30
Ratio MGDG/DGDG ^c (mol/mol)	0.28 (1.07 ^d)	0.35	0.41	0.51	0.62
% ionic lipids ^e of total polar lipids (mol/mol)	48	40	36	35	23
μmol total lipids per μg membrane protein	0.60	1.44	0.86	0.89	0.82

The variability of the values, expressed as $\pm$ S.E. (3), never exceeded 5%. The number of experiments is given within parenthesis.

^a PPLO serum fraction (Difco Laboratories), commercial serum for mycoplasmas acting here as source of oleic (18:1c) and linoleic (18:2c) acid and cholesterol. Palmitic acid was not added.

^b Membrane lipids contained approximately 25% (mol/mol) 18:2c and 60% 18:1c (mol/mol) as determined by gas-liquid chromatography and liquid scintillation counting.

^c Ratio monoglucosyldiglyceride/diglucosyldiglyceride.

^d Ratio (MGDG + cholesterol)/DGDG. These membranes contained 24% (mol/mol total lipids) cholesterol.

^e Ionic lipids: phosphatidylglycerol, glycerophosphoryl-mono- and di-glucosyldiglyceride.

Table III: Solubilization of membranes with different lipid composition^a

Sample no.	Supplementation of palmitic and oleic acid ($\mu\text{M}/\mu\text{M}$) to the growth medium ^b	% in Tw 20 supernatant	% in NaDOC supernatant
I	PPLO ser. fract.	52	89
II	0/150	60	73
III	30/120	88	100
IV	75/75	76	99
V	120/30	81	87

S.E. (5) never exceeded $\pm 10\%$.

^a Percentages refer to concentration of membrane constituents as determined by absorbance measurements at 280 nm in the two detergent supernatants, respectively, as compared to the total concentration in NaDodSO_4 (see Materials & Methods).

^b See Table II for lipid composition of the corresponding membrane.

Table IV: Lipid composition in membranes after extraction with 2.5% (w/v) Tween 20 (1 h, 20°C).
See Table II for symbols and explanations

Sample no.	I	II	III	IV	V
Supplementation of palmitic and oleic acid ($\mu\text{M}/\mu\text{M}$) to the growth medium ^a	PPLO ser. fract.	0/150	30/120	75/75	120/30
% oleic acid in lipids (mol/mol)	_b	95	84	54	20
Ratio MG/DG/DG/DG (mol/mol)	0.89 (1.45 ^c)	0.59	0.86	1.44	1.93
Ratio MG/DG after/ before extraction	2.52	1.34	1.71	1.66	1.34
% ionic lipids of total polar lipids (mol/mol)	40	35	31	25	14
μmol total lipids per μg membrane protein	0.75	1.40	0.77	0.90	0.88

S.E. (3) never exceeded $\pm 5\%$.

^a Cf. Table II.

^b Not determined, cf. Table II.

^c Ratio (MG/DG + cholesterol)/DG/DG. Membrane residue contains 14% (mol/mol total lipids) cholesterol.

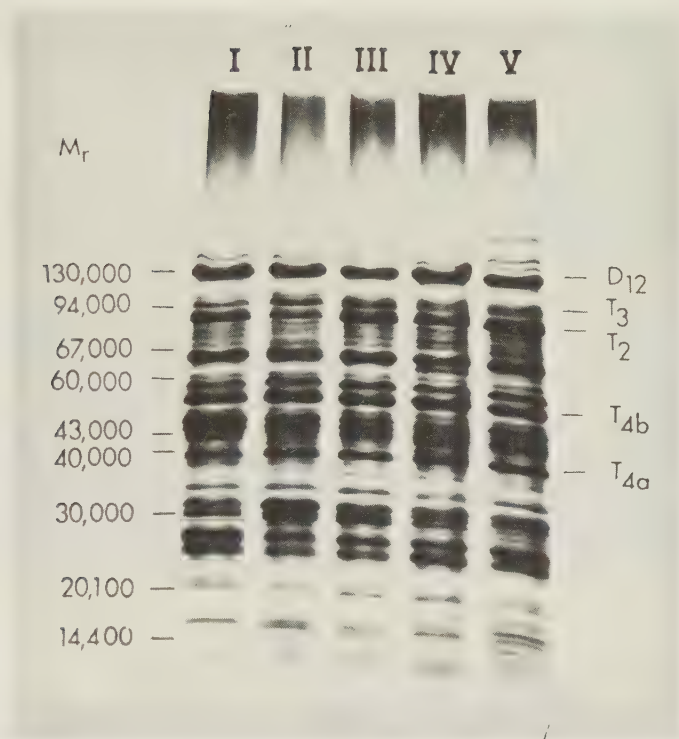


Figure legends

FIGURE 1. NaDodSO_4 polyacrylamide gel electrophoresis (7-22.5% w/v linear acrylamide gradient) of membrane proteins from *A. laidlawii* B(ju) grown with different fatty acid supplements. See Table II for growth medium supplements and membrane lipid composition. The positions shown for individual *A. laidlawii* proteins are those obtained by co-electrophoresis of the purified, single proteins. Mr, molecular weights of reference proteins.

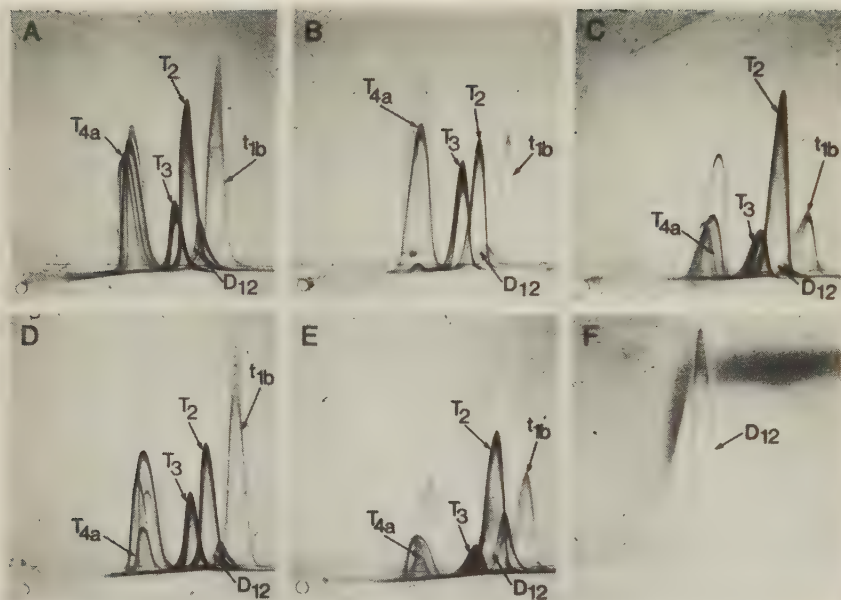


FIGURE 2. CIE of detergent supernatants of membranes from *A. laidlawii* grown with different fatty acid supplements. A-E, Tw 20 supernatants; F, NaDOC supernatant. A, supplement I (see Table II); B, supplement II; C, supplement III; D, supplement IV; E, supplement V. The identities of individual immunoprecipitates in A-E were established by CIE with an intermediate gel containing a monospecific antiserum. F; CIE of a NaDOC supernatant against a monospecific antiserum (anti-D₁₂) in the NaDOC-CIE system.

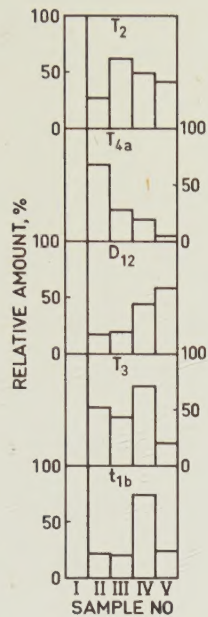


FIGURE 3. Relative amounts of the *A. laidlawii* membrane proteins T₂, T_{4a}, D₁₂, T₃, and t_{1b} in Tw 20 supernatants of the membrane as determined by CIE with a polyspecific antiserum. The sample numbers refer to the fatty acid supplements given in Table II where also the corresponding lipid compositions of the membranes are given. Identification and quantification of individual proteins were done as described in Experimental Procedures. S.E. (3) never exceeded $\pm 8\%$.

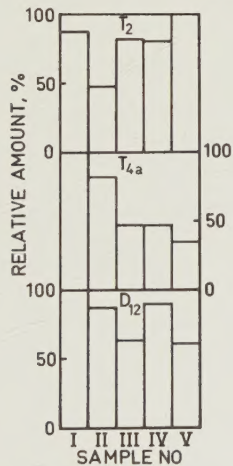


FIGURE 4. Relative amounts of the proteins T₂, T_{4a}, and D₁₂ in NaDOC supernatants of *A. laidlawii* membranes as determined by CIE with monospecific antisera. The sample numbers refer to the fatty acid supplements given in Table II. S.E. (3) never exceeded $\pm 8\%$.

